UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JEFERSON JÚNIOR DA SILVA

BIÓTIPOS DE ESPÉCIES DE *Candida* NA CAVIDADE BUCAL DE CRIANÇAS PORTADORAS DE FISSURAS LABIAL E PALATINA

Candida SPECIES BIOTYPES IN THE ORAL CAVITY OF CHILDREN WITH CLEFT LIP AND PALATE

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Candida SPECIES BIOTYPES IN THE ORAL CAVITY OF CHILDREN WITH CLEFT LIP AND PALATE

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Buco-Dental, na Área de Microbiologia e Imunologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Buco-Dental Biology, in Microbiology and Immunology area.

Orientador: Prof. Dr. Marcelo Fabiano Gomes Boriollo
Este exemplar corresponde à versão final da tese defendida pelo aluno Jeferson Júnior da Silva e orientada pelo Prof. Dr. Marcelo Fabiano Gomes Boriollo

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A ATA de Defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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“Não há limites quando você está rodeado de pessoas que acreditam em você, ou de pessoas cujas expectativas não são definidas por atitudes míopes da sociedade, ou por pessoas que ajudam a abrir portas de oportunidade e não fechá-las”

Neil de Grasse Tyson

RESUMO

Os pacientes com fissuras orofaciais apresentam vários fatores de risco às doenças infecciosas bucais, decorrentes das alterações anátomo-fisiológicas e aquelas resultantes das intervenções terapêuticas reabilitadoras. A incidência das espécies de *Candida* em grupos de bebês e crianças com fissuras orofaciais, durante os períodos pré- e pós-operatórios e até o retorno à primeira consulta, e os perfis de sensibilidade antifúngica e de virulência *in vitro* foram investigados. Amostragens bucais foram coletadas em diferentes tempos ao longo dos procedimentos cirúrgicos e consulta clínica pós-cirúrgica e semeadas em meios de cultivo cromogênico CHROMagar Candida®. Biotipos de *Candida* foram identificados acessando sequências genômicas de DNA espécie-específicas por técnicas de PCR e procedimentos eletroforéticos. Testes de sensibilidade antifúngica foram realizados pelo método de microdiluição em caldo usando os antifúngicos anfotericina B (AP), nistatina (NYS) e fluconazol (FLC). Atividades exoenzimáticas de SAP e PL foram determinadas por métodos microbiológicos clássicos. Algumas fissuras orofaciais ocorreram preferencialmente no sexo masculino ou feminino. Baixa incidência (39,1%) de colonização bucal por espécies de *Candida* (*C. albicans*, *C. krusei*, *C. tropicalis* e *Candida* spp.) foi relatada na admissão do paciente no centro cirúrgico, sem correlação aos tipos de fissuras orofaciais ou histórico cirúrgico. Redução significativa nas frequências de *Candida* e alterações das espécies, ao longo dos períodos de amostragens, revelaram padrões dinâmicos de colonização bucal: eliminação, manutenção ou neocolonização dos biótipos. Esses biótipos mostraram sensibilidade ao AP (100%), resistência parcial ao FLC (<10%) e MICs variáveis para NYS (0,125-4 μg/mL), além de fortes atividades exoenzimáticas, especialmente para SAP. As condutas clínicas e terapêuticas para a reabilitação cirúrgica, as características anatômicas e fisiológicas dos portadores de fissuras orofaciais, e o comportamento cultural e regionalismo da população de pacientes atendida, poderiam influenciar as frequências e a dinâmica de colonização bucal por espécies de *Candida*. Os dados revelaram biótipos de *Candida* resistentes ao FLC e sensíveis (AP) ou clinicamente compatíveis (NYS) aos polienos, especialmente *C. albicans*, na cavidade bucal de pacientes predispostos à colonização bucal e candidoses, contribuindo com as condutas clínicas em eventuais terapias antifúngicas. Esses biótipos foram considerados potencialmente virulentos e capazes de modular parcialmente seus fatores de virulência, especialmente SAP, sob as condições favorecidas pelo hospedeiro.

Palavras chaves: Espécie de *Candida*. Fissura labial e palatina. PCR. Antifúngicos. Virulência.

ABSTRACT

Patients with orofacial clefts present various risk factors for oral infectious diseases, resulting from anatomical and physiological changes and those resulting from rehabilitating therapeutic interventions. The incidence of *Candida* species in groups of babies and children with orofacial clefts, during pre- and post-operative periods and until return to first consultation, and the profiles for antifungal sensitivity and virulence *in vitro* were investigated. Oral samples were collected at different times over the surgical procedures and post-surgical clinical consultation and seeded in chromogenic culture media CHROMagar Candida®. *Candida* biotypes were identified by accessing species-specific genomic DNA sequences by PCR techniques and electrophoretic procedures. Antifungal susceptibility testing was performed by the method of microdilution in broth using the antifungals amphotericin B (AP), nystatin (NYS) and fluconazole (FLC). SAP and PL exoenzyme activities were determined by classical microbiological methods. Some orofacial clefts occurred preferentially in male or female. Low incidence (39.1%) of oral colonization by *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis* and *Candida* spp.) was reported in patient admission to surgical ward, with no correlation to orofacial cleft types or surgical history. Significant reduction in frequencies of *Candida* and changes of species, over sampling periods, showed dynamic patterns of oral colonization: elimination, maintenance or neocolonization of the biotypes. These biotypes showed sensitivity to AP (100%), partial resistance to FLC (<10%) and variable MICs for NYS (0.125-4 μg/mL), in addition to strong exoenzyme activities, especially for SAP. Clinical and therapeutic conducts for surgical rehabilitation, anatomical and physiological characteristics of patients with orofacial clefts, and cultural behavior and regionalism of the patient population served could influence the frequencies and dynamics of oral colonization by *Candida* species. The data showed *Candida* biotypes resistant to FLC and sensitive (AP) or clinically compatible (NYS) to polyenes, especially *C. albicans*, in the oral cavity of patients predisposed to oral colonization and candidiases, contributing to clinical conducts in possible antifungal therapies. These biotypes were considered potentially virulent and able to partially modulate their virulence factors, especially SAP, under the conditions favored by host.

Keywords: *Candida* species. Cleft lip and palate. PCR. Antifungals. Virulence.

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LISTA DE ABREVIATURAS E SIGLAS

ACT1	–	Associated intron sequence
AB	–	Anfotericina B
APSs	–	Aspartil proteinases secretadas
ATCC	–	American Type Culture Collection
bp	–	Base pairs
Ca	–	*Candida albicans*
$CaCl_2$	–	Calcium chloride
CB	–	Candidíase bucal
CBS	–	Central Bureau Voor Schimmelcultures
CLSI	–	Clinical Laboratory Standards Institute
CP	–	Cleft palate
CLP	–	Cleft lip and palate
EDTA	–	Ethylenediamine tetraacetic acid
e.g.	–	*exempli gratia* (latim)
FLU	–	Fluconazole
FL	–	Fissura de lábio
FLs	–	Fosfolipases
FP	–	Fissura palatina
FLP	–	Fissura labial e palatina
HCl	–	Hydrochloric acid
I	–	Intermediate
i.e.	–	*id est* (latim)
µg	–	Microgram
µL	–	Microliter
µM	–	Micromolar
mM	–	Millimolar
Na_2EDTA	–	Ethylenediamine tetraacetic acid Disodium Salt

NaCl	–	Sodium chloride
NaH_2PO	–	monosodium phosphate
NaOAc	–	Sodium acetate
NaOH	–	Sodium hydroxide
NIS	–	Nistatina
OH	–	Oral habitat
PBS	–	Phosphate buffered saline
PCR	–	Polymerase chain reaction
PLs	–	Phospholypases
Pz	–	Enzymatic activity
R	–	Resistance
S	–	Sensitive
SDD	–	Sensitive-dose dependent
SAPs	–	Secreted aspartyl proteinases
SDA	–	Sabouraud dextrose agar
SDS	–	Sodium dodecyl sulfate
ST	–	Sorbitol
TBE	–	Tris-borate-EDTA
TE	–	Tris-EDTA
UFC	–	Unidades formadoras de colônia
YEPD	–	Yeast Extract Peptone Dextrose

LISTA DE SÍMBOLOS

♂	–	Símbolo de Marte (gênero masculino)
♀	–	Símbolo de Vênus (gênero feminino)
β	–	Beta
°	–	Grau
μ	–	Mu
#	–	Número
∑	–	Sigma (Somatório)

SUMÁRIO

1 INTRODUÇÃO

Fissuras orofaciais, incluindo fissura de lábio (FL), fissura palatina (FP) e fissura labial e palatina (FLP), são os defeitos congênitos mais comuns na região craniofacial. Eles podem ser encontrados isolados ou como parte de uma síndrome. Fissura do lábio e do palato juntas são cerca de duas vezes mais comuns que as fissuras do lábio ou palato isoladas (Hazza'a et al., 2011). A sua etiologia é complexa e envolve vários aspectos genéticos e ambientais. O desenvolvimento embriológico labial e palatal é bem documentado com o desenvolvimento normal dos lábios ocorrendo entre as semanas 4 e 8 de gestação. Deformidades de lábio, palato e nariz são o resultado da interrupção do desenvolvimento normal. A gravidade é ditada pelo tempo, severidade, e quantidade de interrupção ocorrida (Shkoukani; Chen; Vong, 2013).

A prevalência desta malformação varia de acordo com a região geográfica, grupo étnico e racial, exposição ambiental e status socioeconômico, podendo variar entre 1: 700 e 1: 2500 nascidos vivos (Sousa; Roncalli, 2017). Cerca de 70% de todas as fissuras são classificadas como não sindrômicas, onde nenhum defeito estrutural reconhecível além da fenda é detectado. Os 30% restantes são sindrômicos, onde uma fenda apresenta uma anomalia estrutural definida de forma consistente; estes geralmente de natureza mendeliana (Adeyemo; Butali, 2017).

Segundo dados epidemiológicos, cerca de 7.000 crianças nascidas com fendas orofaciais foram descritas nos Estados Unidos anualmente (Shkoukani; Chen; Vong, 2013). No Brasil, um estudo mostrou que a prevalência de nascidos vivos portadores de fissuras orofaciais entre 2009 e 2013 foram de 5,86 em 10.000 nascimentos, com diferenças entre as unidades federativas. A fissura de palato é o tipo mais comum de fissura orofacial em todos as regiões brasileiras (Sousa; Roncalli, 2017). Além dos efeitos físicos sobre o paciente, também há efeitos psicológicos e socioeconômicos que afetam tanto o paciente como os familiares, incluindo a interrupção do tratamento psicossocial e a diminuição da qualidade de vida. Estes defeitos congênitos estão associados com aumento da mortalidade por muitas causas, incluindo o suicídio, bem como custos substanciais de tratamento (Shkoukani; Chen; Vong, 2013).

A grande variedade clínica de fissuras orofaciais, juntamente com seus muitos graus de gravidade e possíveis associações com outras síndromes ou defeitos congênitos, resulta em diferentes protocolos de tratamento. Em geral, a reabilitação é

um longo processo que inclui cuidados clínicos e cirúrgicos. No Brasil, os cuidados de saúde oferecidos às pessoas com as anomalias craniofaciais são predominantemente financiadas pelo estado e a distribuição geográfica das unidades de saúde para este tipo de procedimento é mal distribuído, com maior concentração na região Sudeste, considerada a mais rica do país. Assim, pacientes em muitos outros locais precisam viajar longas distâncias para obter acesso ao tratamento (Sousa; Roncalli, 2017).

A abordagem do paciente com fissura labial e palatina é multidisciplinar, e a equipe deve estar idealmente composta por cirurgiões craniofaciais, otorrinolaringologistas, geneticistas, anestesiologistas, fonoaudiólogos, nutricionistas, ortodontistas e psicólogos, além de neurocirurgiões e oftalmologistas em casos raros de fendas faciais. Desta forma, é possível fornecer acompanhamento a longo prazo no período de desenvolvimento do paciente (de Ladeira; Alonso, 2012).

Os custos de cuidados de saúde para a reabilitação são geralmente altos. Por outro lado, se essas deficiências não são adequadamente abordadas, eles podem levar a complicações relacionadas a problemas sociais, de alimentação e infecções, principalmente em crianças afetadas por doenças orofaciais (Sousa; Roncalli, 2017). Como objetivos finais do tratamento, a equipe multidisciplinar busca proporcionar ao paciente a estética facial normalizada, a integridade do palato primário e secundário, a fala e audição normais, a permeabilidade das vias aéreas, a oclusão de classe I ou neutroclusão (classificação de Edward Harthey Angle, 1899) com mastigação normal, a boa saúde dentária e periodontal e o normal desenvolvimento psicossocial (de Ladeira; Alonso, 2012).

Crianças portadoras de fissura labial e palatina muitas vezes experimentam problemas de alimentação, deglutição, fala e estéticos, bem como uma saúde bucal deficiente. A comunicação entre o espaço nasofaríngeo e a cavidade bucal em pacientes portadores de fissuras orofaciais pode levar a alteração na microbiota da cavidade bucal (Rawashdeh; Ayesh; Darwazeh, 2011). Este fator colabora para a conversão de microrganismos bucais anfibiontes, isto é, microrganismos capazes de agredir o hospedeiro quando as condições ambientais e imunológicas são favoráveis (Foschi et al., 2006), para desenvolverem-se à forma patogênica, como as espécies de *Candida*.

Espécies de *Candida* são fungos dimórficos e foram isoladas pela primeira vez em 1844 a partir do escarro de um paciente com tuberculose. Como outros fungos, eles são organismos não-fotosintéticos eucarióticos com uma parede celular que fica externa

à membrana plasmática. A membrana plasmática contém grandes quantidades de esteróis, geralmente ergosterol. Eles podem metabolizar a glicose sob a forma aeróbica e em condições anaeróbias (Agrawal et al., 2014).

A candidose bucal é causada principalmente por *Candida albicans*, que representa até 81% dos casos entre indivíduos infectados pelo HIV. Pesquisas revelam que cerca de 17% a 75% dos indivíduos saudáveis podem ser colonizados por espécies de *Candida*. No entanto, as espécies de *Candida* não-*albicans* foram implicadas na colonização da cavidade bucal, eventualmente causando infecção em 20-40% dos indivíduos imunocomprometidos (Mushi et al., 2017).

A infecção por *Candida* está associada a determinados fatores patogênicos. Este está intimamente relacionado a aderência de *Candida* às membranas celulares epiteliais, um importante passo no início da infecção, que é promovido por certos componentes da parede celular fúngica, tais como manose, receptores C3d, manoproteína e sacarinas. Outros fatores implicados são a formação de tubo germinativo, presença de micélio, persistência dentro de células epiteliais, endotoxinas, indução do fator de necrose tumoral e a produção de enzimas hidrolíticas, como proteinases e fosfolipases (Agrawal et al., 2014).

Uma variedade de fatores sistêmicos e locais podem causar um crescimento excessivo de espécies de *Candida* na mucosa bucal, levando a transição de espécies de *Candida* comensais para patogênica (e.g., função danificada da glândula salivar, ausência de proteínas antimicrobianas na saliva, tais como lactoferrina, sialoperoxidase, lisozima e anticorpos específicos que interagem com o microrganismo e evitam o crescimento excessivo de *Candida*) (Mushi et al., 2017). Drogas, como os esteroides, o uso de dentaduras e próteses, câncer de boca / leucoplasia e uma dieta rica em carboidratos influenciam significativamente na colonização de *Candida* (Agrawal et al., 2014).

Existem várias classes de compostos que compõem o arsenal contra infecções por *Candida*. Os polienos, os azóis e as equinocandinas são as principais classes utilizadas. O antifúngico mais comumente utilizado no tratamento dessas infecções é o fluconazol (Whaley et al., 2017). O fluconazol pertence à classe dos azóis e são bastante utilizados na micologia médica, por apresentar amplo espectro de ação. Os azóis apresentam menor toxicidade que os polienos e possuem uma boa biodisponibilidade quando administrados por via oral. Seu mecanismo de ação atua inibindo a biossíntese de ergosterol (Barbosa; Faria, 2014). O uso generalizado do fluconazol resultou no

surgimento de resistência em cepas de *C. albicans* previamente sensíveis e na seleção de espécies de *Candida* intrinsecamente menos sensíveis ao fluconazol (Osaigbovo; Lofor; Oladele, 2017). Este fato, dificulta drasticamente o uso empírico do fluconazol como tratamento para candidoses bucais.

Entre os polienos encontram-se a nistatina e a anfotericina B. Esta classe de fármacos apresenta alta toxicidade e baixa seletividade para o hospedeiro. Ambos possuem o mesmo mecanismo de ação, e atuam inibindo um componente da membrana plasmática dos fungos, o ergosterol (Barbosa; Faria, 2014). A nistatina pode ser administrada por meio de uma variedade de veículos, incluindo creme, unguento, pastilhas ou suspensão oral. As pastilha de nistatina tem ação prolongada e contém glicose, o que piora a diabetes mellitus. A suspensão oral pode causar perturbação gastrointestinal, diminuição da intolerância à glicose e maior risco para cárie. A anfotericina B pode ser indicada sob a forma de pomada, creme ou loção três a quatro vezes por dia durante um máximo de 2 semanas, enquanto que a suspensão oral e a via intravenosa tem sido indicadas para o tratamento de infecções fúngicas sistêmicas (Millsop; Fazel, 2016).

Estimativas globais sugerem que a candidose invasiva ocorre em mais do que um quarto de milhão de pacientes a cada ano, com taxas de incidência para candidemia de 2-14 por 100.000 habitantes baseados em população-estudos. A resistência antifúngica é menos comum em *C. albicans,* mas com uso de antifúngico a longo prazo e com infecções recorrentes, como a candidose orofaríngea recorrente, este perfil vem sofrendo mudanças. Várias espécies de *Candida* não-a*lbicans*, como *Candida krusei*, são intrinsecamente resistentes ou menos sensíveis a várias classes de antifúngicos, enquanto outros, como *Candida glabrata*, desenvolvem resistência adquirida após exposição aos agentes antifúngicos. Embora incomum, cepas resistentes à múltiplas drogas antifúngicas têm sido cada vez mais relatadas (Arendrup; Patterson, 2017). Portanto, investigações epidemiológicas envolvendo grupos de pacientes predispostos aos eventos de colonização e infecção fúngica, como aqueles portadores de fissuras orofaciais, devem ser constantemente realizadas, visando a promoção do conhecimento cientifico e popular, além de promover um tratamento efetivo e o bem estar dos pacientes envolvidos.

2 ARTIGO: *Candida* species biotypes in the oral cavity of babies and children with orofacial clefts under surgical rehabilitation

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Abstract

The incidence of *Candida* species in groups of babies and children with orofacial clefts, during pre- and post-operative periods and until return to first consultation, and the profiles for antifungal sensitivity and virulence *in vitro* were investigated. Oral samples were collected at different times over the surgical procedures and post-surgical clinical consultation and seeded in chromogenic culture media. *Candida* biotypes were identified by PCR techniques. Antifungal susceptibility testing was performed by microdilution method (amphotericin B^{AP}, $nystatin^{NYS}$ and $fluconazole^{FLC}$). Secreted $aspartyl\ proteases^{SAP}$ and $phospholipase^{PL}$ exoenzyme activities were determined by microbiological methods. Some orofacial clefts occurred preferentially in male or female. Low incidence (39.1%) of oral colonization by *Candida*species (*C. albicans*, *C. krusei*, *C. tropicalis* and *Candida* spp.) was reported in patient admission to surgical ward, with no correlation to orofacial cleft types or surgical history. Significant reduction in frequencies of *Candida* and changes of species, over sampling periods, showed dynamic patterns of oral colonization: elimination, maintenance or neocolonization of the biotypes. These biotypes showed sensitivity to AP (100%), partial resistance to FLC (<10%) and variable MICs for NYS (0.125-4mg/mL), in addition to strong exoenzyme activities (SAP). The data showed *Candida* biotypes resistant to FLC and sensitive (AP) or clinically compatible (NYS) to polyenes, especially *C. albicans*, in the oral cavity of patients predisposed to oral colonization and candidiases, contributing to clinical conducts in possible antifungal therapies. These biotypes were considered potentially virulent and able to partially modulate their virulence factors, especially SAP, under the conditions favored by host.

Keywords: *Candida* species. Cleft lip and palate. PCR. Antifungals. Virulence.

Introduction

Congenital orofacial malformations affect the structure and functions of the oral cavity, significantly modifying its characteristics. As a result, such malformations can have influence on the microbiota of the environment. Orofacial clefts are congenital malformations of the middle third of the face that present varying degrees of severity. They are the most common congenital developmental malformations of the oral cavity. This condition adversely affects natural suction or impairs the ability to swallow food. The treatment of these patients is a process that begins at birth and continues into adulthood, involving a multidisciplinary team in order to promote the rehabilitation of the patient. The etiology of these malformations is a controversial topic, possibly multifactorial, on which genetic and environmental factors can act in isolation or in association (1).

Patients with orofacial clefts present various risk factors for oral infectious diseases, resulting from anatomical changes of the maxillary segments, generated by deficient fusion of the facial embryonic processes. This fact is related to poor dental positioning, nasal septum deviation or nostril stenosis, which leads to mouth breathing. Other changes include those resulting from rehabilitating therapeutic interventions, such as scar fibrosis resulting from surgical repair, the use of orthodontic appliances and/or dental prostheses, which modify the ecological environment of the oral microbiota in patients with cleft lip and palate, and may encourage the colonization of the oral cavity by pathogenic microorganisms or make pathogenic the commensal members of this microbiota, including among these the Candida species (2).

Candida is commonly present in the normal oral microbiota of healthy individuals. Its presence is estimated in 45–65% of healthy babies and in 30–55% of healthy adults. In humans, the most common *Candida* species found in the oral cavity is *C. albicans* due to its adhesion properties and greater degree of pathogenicity. *C. albicans* is a dimorphic yeast, which can exist both in forms of yeasts and of hyphae, depending on the environment. *C. albicans* has been isolated in more than 80% of oral lesions. Other species that are incident to oral infections include *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. kefyr*, *C. parapsilosis*, *C. stellatoidea* and *C. tropicalis* (3).

A variety of systemic and local factors can cause overgrowth of *Candida* species in buccal mucosa, leading commensal *Candida* species to become pathogenic,

making oral candidiasis (OC) an important oral dermatologic disease. The factors include the use of dentures, corticosteroid and xerostomia inhalers, while systemic factors include immunosuppressed states, such as the human immunodeficiency virus (HIV), leukemia, malnutrition, impaired immunity related to aging, endocrine dysfunction such as diabetes, anatomical changes, chemotherapy, radiation therapy, and the use of systemic corticosteroids, immunomodulatory drugs, xerogenic drugs and broad-spectrum antimicrobials (3).

The transition from amphibiont to pathogenic form in *Candida* spp. has been attributed to the selective expression of various virulence factors, which act synergistically, under favorable predisposing conditions (4). Thus, the type, stage and site of the infection, as well as the nature of individual immune response, cause the yeast to express one or more virulence factors (5). Among the virulence factors, extracellular enzyme activity, proteolytic or lipolytic, plays important role in the pathogenicity of *C. albicans* (6,7). Lipolytic enzymes have an active role in the invasion of lesions in the host tissue, since these enzymes cause rupture of the epithelial cell membrane and allow fungi cell to penetrate the cytoplasm. While proteolytic enzymes induce the degradation of a wide variety of host proteins, facilitating fungi penetration into the tissues (8).

There are several classes of compounds that constitute the arsenal against numerous *Candida* infections. Polyenes, azoles, echinocandins and alilamins are used, varying according to the type of infection and the sensitivity of the *Candida* species involved (9). The antifungal prescription most often employed against infections caused by *C. albicans* has been fluconazole, a member of the class of azoles. However, cases of infections by fluconazole-resistant *Candida* species have also been reported. Clinical isolates of *C. albicans* from candidemic patients have the lowest incidence of resistance to azoles (0–5%) among the Candida species (9). Azoles are fungistatic against *Candida* spp. and act by binding and inhibiting the intracellular target enzyme ERG11p, involved in ergosterol biosynthesis. More than 140 alterations were described in target gene ERG11, some of which were found exclusively in azole-resistant isolates, while others were also found in sensitive isolates. Moreover, efflux pumps contribute significantly to azole resistance in *Candida* spp. (10).

Various systemic and topical agents are currently available for the treatment of oral candidiasis. Systemic antifungal agents, including fluconazole and itraconazole, are suitable for patients who do not respond to or are intolerant to topical treatment

and who have high risk of developing systemic infections. However, numerous drug interactions and the decrease in sensitivity limit the application of systemic antifungal agents. Topical antifungal agents, such as nystatin and amphotericin B, are typically recommended for first-line treatment for cases of oral candidiasis (11). Amphotericin B has fungicidal activity through its binding to ergosterol, present in the fungal cell membrane. This association results in the formation of pores in the membrane and the loss of intracellular compounds and cell death. *Candida* spp. mutation mechanisms resulting in reduced binding between ergosterol and amphotericin B establish antifungal resistance. However, the mechanisms that establish resistance to amphotericin B and nystatin are considered rare (10). Therefore, the detection of pathogenic fungal strains resistant to antifungal therapy in patients predisposed to colonization or infection processes, such as patients with orofacial clefts, is essential for effective treatment, clinical health and welfare of patients (12).

Based on data from the available literature and aiming to contribute to studies on the epidemiology of *Candida* species and their intrinsic characteristics of pathogenicity, this research investigated (*i*) the incidence oral clinical *Candida* species from babies and children with orofacial clefts, before, during and after surgical rehabilitation procedures, and their possible epidemiological and clinical correlations, (*ii*) the characteristics of virulence *in vitro* of Candida species, especially the hydrolytic enzymes secreted aspartyl proteinases (SAP) and phospholipases (PL), and (*iii*) the antifungal sensitivity and resistance patterns; the minimum inhibitory concentration (MIC) *in vitro* of the antifungal agents amphotericin B, nystatin and fluconazole.

Material and methods

Subjects

The study involved 46 patients, aged between 0 and <12 years, both male and female, with orofacial clefts, indicated for surgical rehabilitation, under medical and dental follow-up in the Clinics of the School of Dentistry of the José do Rosário Vellano University (UNIFENAS) — Centro Pró-Sorriso aos Portadores de Fissuras Labial e Palatina —, of the municipality of Alfenas, state of Minas Gerais, Brazil. Patients showing

different types of orofacial clefts (13) with and without surgical history were subdivided into two main groups: Babies (*n* = 27; mean of 11.2 ±6.6 months of age) and children (*n* = 19; mean of 7.2 ±3.2 years of age) (Figure 1 and Table 1). This research was conducted in accordance with Resolution No. 466/2012 of the National Health Council and approved by the Research Ethics Committee of the FOP/UNICAMP (Protocol No. 093/2014).

Sampling

Microbiological samples were obtained using the method described previously by Samaranayake et al. (1986) (14), with some adaptations. For each patient with orofacial cleft, the samples were collected (pre-surgery: orofacial clefts; post-surgery: oral cavity rehabilitated surgically) using a sterile swab, in the presence of a physician, and maintained in 50 mL polypropylene tubes containing 10 mL of sterile PBS solution (100 mM NaCl, 100 mM NaH_2PO_4, pH 7.2). Then, these samples were properly transported (4°C) to the Laboratory of Oral Microbiology and Immunology, School of Dentistry of Piracicaba, State University of Campinas (FOP/UNICAMP). These tubes were centrifuged at 1,700 × g for 10 minutes, the supernatant was discarded and the sediments were resuspended in 1 mL of sterile PBS solution (concentrated sample 10×). Soon after, the sediments were transferred to 2 mL microtubes and shaken in vortex for 0.5 minute (14,15). Then, 100 μL aliquots of each sample were inoculated on plates containing CHROMagar Candida® (1.5% agar, 1.02% peptone, 2.2%, chromogenic mixture, 0.5% chloramphenicol) (16,17) and aerobically incubated at 37°C for 48 hours (18,19). For each patient with orofacial cleft microbiological samples were obtained at 4 different times between hospitalization (2 days) and return (18–184 days, mean of 52.8 ±49.9 days) of patients, in the period from August 2015 to July 2016: (A) admission to surgical ward prior to asepsis, (B) prior to surgical procedure and immediately after asepsis with PVP-I or chlorhexidine, (C) immediately after surgical rehabilitation and (D) at the first patient return to the Centro Pró-Sorriso aos Portadores de Fissuras Labial e Palatina, Alfenas, MG, Brazil. The antibiotic therapy prescribed was administered with first-generation cephalosporins (cefazolin or cephalothin) during 7 days from hospitalization (Figure 1).

Preliminary identification of yeasts

The preliminary identification of *Candida* species was based on the color of the colonies on the chromogenic culture medium CHROMagar Candida®, in order to determine the amount (CFU/mL) and quality (mixed or homogeneous cultures) (18–20). This chromogenic medium is a differential culture medium developed for the isolation and presumptive identification of some clinically important *Candida* species: *C. albicans* (distinct green colonies), *C. tropicalis* (distinct dark grayish blue colonies with a dark brownish purple halo in the surrounding agar) and *C. krusei* (highly rugous characteristic, scattered colonies with pale pink centers and a white border). In addition, CHROMagar Candida® has been recommended for the preliminary identification of Candida species most commonly isolated from clinical materials, given the quick and easy recognition of mixed yeast cultures; this can be used as an indicator of populational homogeneity (20). Up to five colonies of each color were selected at random by sampling, cultivated in neutral Sabouraud dextrose agar slant medium (Emmon's modification: 2% glucose, 1% peptone, 2% agar, pH 6.8 to 7.0) and kept at 37°C for 24–48 hours to obtain pure cultures (21). These isolates were stored (*i*) in tubes containing 5 mL of neutral Sabouraud dextrose agar slant medium at –4°C (21) and (*ii*) in tubes containing 3 mL of Sabouraud dextrose broth medium and sterile glycerol (final concentration of 20%) at –70°C (22,23), for short and long periods, respectively. Such procedures enabled accessing these cultures in the course of the analyses. The molecular identities of the yeasts were confirmed by species-specific PCR methods: *C. albicans* (24), possibly *C. dubliniensis* (25), *C. krusei* and *C. tropicalis* (26).

Genomic DNA purification

Genomic DNA of the isolates were obtained using previously described methods (27). Cells of yeasts were cultivated in 50 mL of YEPD medium (1% m/v yeast extract, 2% m/v peptone, 2% m/v D-glucose) at 150 r.p.m (Incubator Shaker, model SL 222, SOLAB Equipamentos para Laboratórios, Piracicaba, SP, Brazil) at 37°C for 18 hours. Soon after the growth, the colonies were transferred aseptically to 2 mL eppendorf tubes and washed twice in 1 mL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 7.5) and twice in 1 mL of ST solution (1.2 M Sorbitol, 10 mM Tris-HCl) submitting

each wash to a centrifugal force of 1,500 × g (Centrifuge, model 5810R, Eppendorf do Brasil Ltda., São Paulo, Brazil) for 5 minutes. The cellular sediments were mixed in 500 μL of lysis solution (100 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA, 1% p/v SDS, 500 μg/mL of proteinase K) and incubated at 37°C for 60 minutes, 65°C for 15 minutes and at room temperature for 10 minutes (Eppendorf® Digital Termomixer). Equal volumes of 1:1 phenol-chloroform were added to the tubes and homogenized several times by inversion movements. These mixtures were centrifuged at 12,000 × *g* (Centrifuge, model 5417R, Eppendorf do Brasil Ltda., São Paulo, Brazil) for 10 minutes and the resulting upper aqueous phases were transferred to new 1.5 mL tubes containing equal volumes of chloroform. Again, these tubes were homogenized, centrifuged at 12,000 × *g* for 10 minutes and the resulting upper aqueous phases were transferred to new 1.5 mL tubes. Genomic DNAs were precipitated by the addition of 2 volumes of ethanol at –20°C for 30 minutes. These DNAs were centrifuged at 12,000 × g for 10 minutes, dried at 30°C for 90 minutes and dissolved in 500 μL of TE buffer added with 500 μg/mL RNAse A, where they were kept at 37°C for 30 minutes. Again, DNAs were extracted once with 1:1 phenol-chloroform and once with chloroform, precipitated with 0.04 volume of 2M NaOAc and 2 volumes of ethanol at –20°C for 30 minutes, centrifuged at 12,000 × *g* for 10 minutes, dried at 30°C for 90 minutes and dissolved in 500 μL of TE buffer. These solutions were stored at –20°C until the moment of use. DNA quantification for each isolate was performed by Spectrophotometric dosage at 260 nm (Thermo Scientific NanoDrop® 2000c Spectrophotometer, Wilmington, DE, USA).

Primers and PCR

The pairs of species-specific primers for *C. albicans* OK3 (*forward* 5´ - ATG TAT TCA TTA ATC AAA TCA - 3´) and OK4 (*reverse* 5´ - ATT TAA AAA ACA ACG GAC AT - 3´) were employed to amplify a DNA fragment of approximately 1,644 bp (*PHR1* gene) (19,24). The amplification reactions were performed in a final volume of 40μL containing PCR Supermix (Invitrogen ™, Cat. # 10572-152, Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.160 μM of each primer and 200 ng of genomic DNA. Amplifications were conducted in a thermal cycler (Veriti™ Dx 96-well Fast Thermal Cycler, Cat. # 4452299, Applied Biosystems™ PCR instruments, Thermo Fisher Scientific

Inc., Waltham, MA, USA) using an initial program for DNA denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 20 seconds (denaturation), 50°C for 1 minute (annealing) and 72°C for 1.5 minute (elongation). The final cycle was conducted at 72°C for 10 minutes for the final elongation.

The pairs of species-specific primers for *C. krusei* CKSF35 (*forward* 5´ - GAG CCA CGG TAA AGA ATA CAC A - 3´) and CKSR57 (*reverse* 5´ - TTT AAA GTG ACC CGG ATA CC - 3´) were employed to amplify a DNA fragment of approximately 227 bp (*DNA topoisomerase II gene*) and the pairs of species-specific primers for *C. tropicalis* CTPIIF36 (*forward* 5´ - CTG GGA AAT TAT ATA AGC AAG TT - 3´) and CTPIIR121 (*reverse* 5´ - TCA ATG TAC AAT TAT GAC CGA GTT - 3´) were employed to amplify a DNA fragment of approximately 860 bp (*DNA topoisomerase II gene*) (26). The amplification reactions were performed in a final volume of 40μL containing PCR Supermix (Invitrogen ™, Cat. # 10572-152, Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.160 μM of each primer and 200 ng of genomic DNA. Amplifications were conducted in a thermal cycler (Veriti™ Dx 96-well Fast Thermal Cycler, Cat. # 4452299, Applied Biosystems™ PCR instruments, Thermo Fisher Scientific Inc., Waltham, MA, USA) using an initial program for DNA denaturation at 96°C for 2 minutes, followed by 30 cycles at 96°C for 30 seconds (denaturation), 57°C for 30 seconds (annealing) and 74°C for 1 minute (elongation). The final cycle was conducted at 74°C for 1 minute for the final elongation.

Agarose gel electrophoresis and photodocumentation

Amplified PCR products were mixed with 6X DNA Loading Dye (Cat. # R0611, Thermo Fisher Scientific Inc., Waltham, MA, USA), containing SYBR™ Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO, Cat. # S11494 Invitrogem ™, Thermo Fisher Scientific Inc., Waltham, MA, USA), and separated in agarose gels at 1.5% (m/v) (UltraPure™ Agarose, Cat. # 16500500, Thermo Fisher Scientific Inc., Waltham, MA, USA), previously prepared with 0.5× TBE buffer solution (5× TBE: 445 mM Tris-base, 445 mM boric acid and 10 mM EDTA). Electrophoresis was conducted in horizontal and continuous system containing 0.5× TBE buffer. 20μL aliquots of each product were applied to the gel and submitted to a voltage of 1.5 volts.cm^{-1} for 4 hours (PowerPac™ Universal Power Supply, Bio-Rad Laboratories Inc., Hercules, CA, USA).

As positive control and to ensure reproducibility of the results, products amplified from genomic DNA of *Candida albicans* (Robin) Berkhout (ATCC® 90028™) — *reference strain* —, *Candida tropicalis* CBS94 and *Candida krusei* CBS 573 — *type-strains* — were systematically applied to the ends and center of each gel next to molecular mass markers (DirectLoad™ PCR 100 bp Low Ladder, SIGMA Cat. # D3687 (19,24,26). After the electrophoretic procedures, the gels were visualized and photodocumented in Kodak Gel Logic 200 Imaging System device (Eastman Kodak Company).

Antifungal susceptibility testing

The determination of minimum inhibitory concentration (MIC) was performed by the method of microdilution in broth following the guidelines established by the *Clinical and Laboratory Standards Institute* (28), employing the antifungals amphotericin B (AB – polyene; CAS # 1397-89-3; Potency 750 μg/mg; Sigma chemical co. and Cat. # A2411) fluconazole (FL – triazolic; CAS # 86386-73-4; Sigma chemical co. and Cat. # F8929) and nystatin (NYS – polyene macrolide; CAS #1400-61-9; Potency ≥4,400 IU/mg → 1 IU corresponding to 0.0002059 mg; Calbiochem and Cat. # 475921). Standard solutions of antifungals were prepared, sterilized by filtration (Millipore Corporation, hydrophilic PVDF Durapore® membrane filter, 0.22 μm, ∅ 47 mm, cat. # GVWP 047 00) and stored in sterile 50 mL polypropylene tubes at a temperature of –70°C. These tests were carried out in triplicate test systems using sterile, disposable microdilution plates, with multiple wells (96-well cell culture microplates, flat-bottom, Corning Inc., N.Y.), containing 100 μL of RPMI-1640 culture medium per well [1.04% m/v RPMI-1640 (with glutamine and phenol red, without bicarbonate: sterilization by filtration), 165 mM MOPS (3-(N-morpholino) propanesulfonic acid; sterilization by filtration), 2% m/v glucose, pH 7.0 at 25°C adjusted with 1 M NaOH)] and 100 μL of RPMI-1640 broth added with the antifungals tested at concentration of 2× (amphotericin B and nystatin: from 0.0625 to 32 μg/mL, fluconazole: from 0.25 to 128 μg/mL).

Prior to testing, the yeasts were cultivated in Sabouraud Dextrose Agar (SDA) at 35°C for 24 hours. Then, an inoculum (10 μL loop) of each sample was added in 5 mL of sterile saline solution (145 mM NaCl) and adjusted to a turbidity of 0.5 McFarland

standard (1×10^6 to 5×10^6 CFU.mL^{-1}) or equivalent to a transmittance of 79.4–83.1% using spectrophotometer with wavelength of 530 nm (T = 79.4%–83.1% → $A_{530\ nm}$ = 2 – $\log_{10}$ %T → $A_{530\ nm}$ = 0.100–0.080). This cell suspension was shaken in Vortex for 15 seconds and diluted 1:50 in sterile saline solution (2×10^4 to 1×10^5 CFU.mL^1), followed by a new 1:20 dilution in RPMI-1640 liquid medium (1×10^3 to 5×10^3 CFU.mL^{-1}), in order to produce the working inoculum [2×]. During the tests, 100 µL aliquots of each working inoculum [2×] were inserted in the wells of the microdilution plates, containing 100 µL/well of RPMI-1640 medium added with the antifungals (final concentration of inoculum equal to 0.5×10^3 to 2.5×10^3 CFU.mL^1), previously prepared as described above. Then, these microdilution plates were incubated at 35°C for 24 hours.

After the incubation period, 70 µL aliquots of triphenyltetrazolium chloride solution (5 mg/mL of type I water, sterilized by filtration; *TPTZ*, *TTZ*, *Tetrazolium Red* or *TTC solution*: 2,3,5-Triphenyltetrazolium chloride, SIGMA Cat. # T8877) were aseptically added to the wells of these plates and, again, incubated at 35°C for 24 hours. This solution has been recommended for the detection of microbial growth in numerous studies by the tetrazolium-formazan biotransformation system, e.g., by dehydrogenase and reductase enzymes: *2,3,5-Triphenyltetrazolium chloride* (colorless) → –2H → *1-Phenyl-2-[(Z)Phenyl(phenyl hydrazono)methyl] diazene* (dark red color) (29). The interpretation of the breakpoints was performed following the guidelines established by the *Clinical and Laboratory Standards Institute* (28). The strains of *Candida krusei* (Castellani) Berkhout — *Issatchenkia orientalis* Kudrjanzev (ATCC® 6258-MINI-PACK™), *type strain* — and of *Candida albicans* (Robin) Berkhout (ATCC® 90028™) — *reference strain* — were used in the tests as reference strains and as quality control strains, respectively.

Virulence **in vitro**

The virulence of *Candida* isolates was determined by testing the production of hydrolytic exoenzymes, aspartyl proteinases (secreted aspartyl proteinases - SAPs) and phospholipases (PLs), in accordance with methods described previously (18,19,30). Briefly, samples of recently cultivated yeasts were resuspended in 1 ml of NaCl (150 mM) and standardized to a concentration of 10^7 CFU.mL^{-1}. Then, 5µL aliquots of each

cell suspension were inoculated in culture medium A (Bovine Serum Albumin Fraction V 1% m/v, Yeast Nitrogen Base 0.15% m/v, Glucose 2% m/v, Agar 2% m/v) and culture medium B (Peptone 1% m/v, Glucose 3% m/v, NaCl 5.7% m/v, $CaCl_2$ 0.06% m/v, Agar 2% m/v, Egg Yolk 5% v/v), following the incubation at 37°C for 7 days (culture medium A) and 4 days (culture medium B). *In vitro* exoenzyme activity, named *Pz* (exoenzyme activity zone), was determined through the formation of a halo around the colonies of yeast and the readings were interpreted according to the formula: $Pz = \frac{dc}{dc + zp}$, where *dc* corresponds to diameter of colony (mm) and *zp* corresponds to external diameter of zone of precipitation (mm). The results were interpreted as follows: a) Pz = 1: absence of enzyme activity (index 0); b) $1 > Pz \geq 0.64$: positive enzyme activity (index 1); and c) $Pz < 0.64$: strongly positive enzyme activity (index 2). These tests were performed in duplicate testing system.

Statistical analyses

The results obtained in this research were submitted to Fisher and Chi-square ($\alpha = 0.05$) tests using computational software BioEstat version 5.0 (31).

Results

Orofacial clefts versus groups of patients and sexes

A total of 11 types of orofacial clefts (*cleft bilateral transforaminal*, *cleft left transforamen*, *cleft post-foramen full*, *cleft post-foramen incomplete*, *cleft pre-foramen full right*, *cleft pre-foramen left complete*, *cleft pre-foramen left incomplete*, *cleft pre-foramen right incomplete*, *cleft submucosa post-foramen incomplete*, *cleft transforamen right* and *deformity groove-gingival*) were observed in the 46 patients, with 25 patients having clinical history of orofacial surgical rehabilitation, served in the Clinics of the School of Dentistry, José do Rosário Vellano University (UNIFENAS) — Centro Pró-Sorriso aos Portadores de Fissuras Labial e Palatina —, of the municipality of Alfenas, state of Minas Gerais, Brazil, in the period from August 2015 to July 2016. Among the 27 infants (mean

of 11.2 ±6.6 months of age), 22 presented a single type of orofacial cleft (18 male and 4 female) and 5 presented two types of orofacial clefts (4 male: CLP11, CLP42, CLP45 and CLP50; and 1 female: CLP33). In addition, among the 19 children (mean of 7.2 ±3.2 years of age), 17 presented a single type of orofacial cleft (11 male and 6 female) and 2 presented two types of orofacial clefts (exclusively male: CLP34 and CLP39) (Table 1).

Of these clinically classified orofacial clefts, 9 and 8 types were observed in the groups of infants and children, respectively, with 3 types exclusive to the group of infants (*cleft post-foramen full*, *cleft pre-foramen left incomplete* and *deformity groove-gingival*) and 2 types exclusive to the group of children (*Cleft pre-foramen full right* and *Cleft submucosa post-foramen incomplete*). However, no statistically significant differences ($p < 0.05$; $p < 0.001$) were observed in the frequencies of certain types of orofacial clefts and between the groups of infants or children (Table 2).

In addition, 8 types of orofacial clefts were observed in each sex, with 3 types exclusive to the male (*cleft bilateral transforaminal*, *cleft post-foramen full* and *cleft pre-foramen right incomplete*) and 3 types exclusive to the female (*cleft pre-foramen full right*, *cleft submucosa post-foramen incomplete* and *deformity groove-gingival*). In these cases, statistically significant differences ($p < 0.05$; $p < 0.001$) were observed in the frequencies of certain types of orofacial clefts and between the male (higher frequency: *cleft left transforamen* and *cleft transforamen right*) and female (higher frequency: *cleft post-foramen incomplete* and *cleft pre-foramen left complete*) (Table 2).

Candida *species in period A*

Oral samples from 46 patients with orofacial clefts were investigated as to the presence of *Candida* species, at 4 different times between hospitalization and patient return (called periods A, B, C and D), employing the chromogenic culture medium (CHROMagar Candida®) for isolation and preliminary identification of the yeasts and the molecular technique of species-specific PCR (Figure 2).

In period A (admission to surgical ward prior to asepsis and antibiotic therapy), *Candida* species were observed in 18 patients (39.1%), colonizing the oral sites in mixed (only one child: CLP1; *C. krusei* and *C. tropicalis*: < 350 CFU/mL) and, mainly, homogenous forms. Three species were identified: *C. albicans* (n = 7; 15.2%; ≥ or < 350 CFU/mL), *C. tropicalis* (n = 8; 17.4%; ≥ or < 350 CFU/mL) and *C. krusei* (n = 4;

8.7%; ≥ or < 350 CFU/mL). Only one patient showed suspected clinical manifestation of pseudomembranous candidiasis (CLP40: *C. tropicalis*, ≥ 350 CFU/mL).

Despite the frequency equivalent to 39.1%, no statistically significant differences ($p < 0.05$; $p < 0.001$) were observed in the frequencies of the *Candida* species and certain types of orofacial clefts or surgical history. However, these frequencies of oral colonization by *Candida* species were significantly higher ($p < 0.05$; $p < 0.001$) for the group of infants ($n = 13$; 28.3%) and males ($n = 14$; 30.4%), regardless of the surgical clinical histories (Table 3 and Figure 1).

Candida *species between periods A and C*

Medical-hospital procedures for orofacial surgery rehabilitation in infants and children had variable times, depending on the complexity of each case. In general, this time ranged from 2 to 3 hours between admission to surgical ward (period A) and surgical rehabilitation (period C). Between these two times (periods A and C), there was a sampling immediately after asepsis with PVP-I or chlorhexidine (period B; 1–2 minutes post asepsis) showing an experimentally nonexisting incidence of *Candida* species in the 46 patients with orofacial clefts. The frequencies of *Candida* species in the patients with orofacial clefts was reduced between periods A and C, after (2 to 3 hours) the procedures of asepsis with chlorhexidine ($p < 0.001$) and PVP-I, and associated to the surgical procedures (cheiloplasty: $p < 0.001$; and palatoplasty: $p < 0.05$) and antibiotic therapy ($p < 0.001$). These results also showed that this reduction may be influenced by potential exogenous contamination of those yeasts, since the *Candida* species in two patients (CLP37: prophylaxis with chlorhexidine; and CLP15: prophylaxis with PVP-I) of period C do not come from period A. Other data showed qualitative (species-specific) and quantitative (CFU/mL) maintenance of *Candida* species between periods A and C: infants CLP43 and CLP48 (homogeneous colonization by *C. albicans*) and CLP45 (homogeneous colonization by *C. tropicalis*) (Table 4 and Figure 1).

Candida *species between samplings*

The frequencies of *Candida species* showed statistically significant differences ($p < 0.001$) between the 4 sampling times. Higher frequency of those species was

observed at admission of orofacial cleft patients to surgical ward (period A: 39.1%), followed by patient return to medical unit (period D: 21.7%; ≥5 and ≤184 days, mean of 52.8 ±49.9 days), rehabilitation orofacial surgery (period C: 10.9%) and immediate asepsis with PVP-I or chlorhexidine in the surgical ward (period B: 0%). Quantitative (%) and qualitative (species-specific) differences were observed between the sampling periods (Table 5 and Figure 1):

1) Period A: homogeneous colonization by *C. albicans*, *C. krusei* or *C. tropicalis*, and mixed colonization by *C. krusei* and *C. tropicalis*;

2) Period C: homogeneous colonization by *C. albicans*, *C. krusei* or *C. tropicalis*, and mixed colonization by *C. krusei* and *C. tropicalis*; and

3) Period D: homogeneous colonization by *C. krusei* or *C. tropicalis*, mixed colonization by *C. albicans* and *C. tropicalis* or *C. albicans* and *C. krusei*, and colonization by other *Candida* spp.

Therefore, a dynamic pattern of oral colonization by *Candida* species, in patients with orofacial clefts, was significantly variable ($p < 0.001$) over the pre- and post-operative experimental periods (Table 6 and Figure 1):

1) Absence of colonization by *Candida* species in the sampling periods (47.8%);

2) Elimination of colonization by *Candida* species subsequent to period A (21.7%: patients CLP21, CLP22, CLP27, CLP33, CLP36, CLP47, CLP50, CLP52, CLP53 and CLP54);

3) Elimination of colonization by *Candida* species subsequent to period A followed by oral neocolonization in period D (10.9%: CLP29 – convergent neocolonization; CLP1 – partially convergent neocolonization; CLP9 and CLP40 – divergent neocolonization; and CLP57 – convergent and divergent neocolonization);

4) Maintenance of colonization by *Candida* species in periods A and C (6.5%; CLP43, CLP45 and CLP48);

5) Neocolonization in period C (CLP37) or period D (CLP2, CLP17, CLP24 and CLP44) (10.9%); and

6) Neocolonization in period C (CLP15) and maintenance and neocolonization in period D (2.2%).

Antifungal susceptibility (MIC)

Antifungal susceptibility tests *in vitro* were performed according to CLSI guidelines (28) using the antifungals Amphotericin B (AP – polyene), Fluconazole (FL – triazolic) and Nystatin (NYS – polyene macrolide). Up to five isolates per patient positive for *Candida* species, of all sampling periods (A, B and D), were submitted to the test. A total of 182 oral clinical isolates of yeasts of the genus *Candida* were investigated, being 60 isolates of *C. albicans*, 60 isolates of *C. krusei*, 57 isolates of *C. tropicalis* and 5 isolates of *Candida* spp. All Candida species were sensitive to amphotericin B (MIC < 1 μg/mL). The frequencies of resistance to fluconazole (MIC ≥ 64 μg/mL) were in the order of 3.3% for the isolates of C. albicans, 8.3% for *C. krusei*, 5.3% for *C. tropicalis* and 0% for *Candida* spp. However, the frequency of dose-dependent sensitivity to fluconazole (MIC 16–32 μg/mL) was 48.3% exclusively for *C. albicans*. MICs for nystatin were variable between the *Candida* species (0.125–4 μg/mL). Higher amplitude of MIC was observed among the isolates of *C. krusei* (0.125–4 μg/mL), followed by *C. albicans* (0.5–2 μg/mL), and *C. tropicalis* and *Candida* spp. (0.5–1 μg/mL) (Table 7).

Virulence potential (SAP and PL)

Virulence potential tests *in vitro* were also investigated among the 182 oral clinical isolates of yeasts of the genus *Candida* (Table 8). Strongly positive activities (*Pz* = 2) of secreted aspartyl proteinases (SAP) were highly frequent in the isolates of *C. albicans* (95%), *C. krusei* (90%), *C. tropicalis* (98.2%) and *Candida* spp. (100%). Between indexes (*Pz*) 1 and 2 for SAP activities, index 2 occurred exclusively in the *Candida* species. The values for *Pz* ranged from 0.29 to 1.00 (mean of 0.43 ±0.14) for the isolates of *C. albicans*, from 0.26 to 1.00 (mean of 0.45 ± 0.19) for *C. krusei*, from 0.31 to 1.00 (mean of 0.43 ±0.08) for *C. tropicalis* and from 0.3 to 0.44 (mean of 0.34 ±0.06) for *Candida* spp.

Strongly positive phospholipase (PL) activities (*Pz* = 2) were proportionately lower or infrequent in the *Candida* species: only for the isolates of *C. albicans* (36.7%) and *C. tropicalis* (5.3%). Between indexes (*Pz*) 1 and 2 for SAP activities, index 2 occurred exclusively in the two *Candida* species. The values for *Pz* ranged from 0.41 to

1.00 (mean of 0.80 ±0.27) for the isolates of *C. albicans* and from 0.47 to 1.00 (mean of 0.97 ± 0.11) for *C. tropicalis*.

Discussion

Invasive infections caused by *Candida* species also contribute to significant rates of morbidity and mortality of hospitalized and immunosuppressed patients. These species are ranked as the fourth leading cause of bloodstream infections, remaining behind the coagulase-negative staphylococci (CoNS), *Staphylococcus aureus* and *Enterococci* (32). Oral candidiasis has been defined as an opportunistic infection of high frequency and clinical relevance, caused by overgrowth of *Candida* species, especially *C. albicans*. Epidemiological information also shows that the mean rate for oral prevalence of *Candida* species corresponds to 17.7% (ranging from 2% to 71%) among healthy individuals (33).

A total of 46 patients aged 0 to <12 years, both male and female, subdivided into two groups (n = 27 infants; n = 19 children), with orofacial clefts (CBT, CLT, CP-FF, CP-FI, CP-FFR, CP-FLC, CP-FLI, CP-FRI, CSP-FI, CTR and DG-G), with (54.3%) and without (45.7%) surgical history, were submitted to surgical procedures of rehabilitation and assessed as to the presence of oral *Candida* species at four different times (called periods A, B, C and D) (Figure 1 and Tables 1 and 2). Of these patients, 5 infants and 2 children presented two types of orofacial clefts (infants: CLP11 with CP-FLI and CP-FI, CLP42 with CP-FRI and CP-FI, CLP45 with CTR and CP-FLI, CLP50 with CLT and CP-FRI, and CLP33 with CP-FLI and CP-FI; children: CLP34 with CP-FRI and CP-FI, and CLP39 with CLT and CP-FRI). Although 3 of 9 types of orofacial clefts are exclusive to the group of infants (CP-FF, CP-FLI and DG-G) and 2 of 8 types are exclusive to the group of children (CP-FFR and CSP-FI), the clinical form of these clefts remained without correlation with the stratified groups of patients. On the other hand, of the 8 types of orofacial clefts observed in each sex, 3 types were exclusive to males (CBT, CP-FF and CP-FRI) and 3 types were exclusive to females (CP-FFR, CSP-FI and DG-G), also being observed correlation of clinical forms CLT and CTR with males and CP-FI and CP-FLC with females (Tables 1 and 2). After the surgical procedures and the hospitalization period, most of these patients returned for the first medical consultation for clinical and

laboratory evaluation, considering a wide variation in the period until return (18 to 184 days, mean of 52.8 ±49.9 days) (Figure 1). Therefore, it is speculated that several cultural, socioeconomic, and even geographical factors of the population served have influence on the conduct of patients served.

Candida species were observed in 18 patients (39.1%) admitted to the surgical ward (period A), colonizing the oral sites in the mixed form (*C. krusei* and *C. tropicalis*; < 350 CFU/mL) and homogeneous form (*C. albicans*, *C. tropicalis* and *C. krusei*; ≥ or < 350 CFU/mL). Therefore, the density of this homogeneous oral colonization was shown to be variable (≥ or < 350 CFU/mL) in the groups of patients with orofacial clefts without clinical symptoms of candidiasis (Figure 1 and Table 3). These data also corroborate the hypothesis that the abundance of organisms in saliva does not necessarily correlate with the forms of oral candidiasis (18). Clinical suspicion for manifestation of pseudomembranous candidiasis occurred only in one patient (*C. tropicalis*; ≥ 350 CFU/mL) in period A, but without any complication of clinical relevance after surgery (Figure 1). The incidence of *Candida* species observed (39.1%) was lower than in the previous studies in groups of patients with orofacial clefts (33). It is believed that these results are influenced by the multidisciplinary professional guidelines and follow-ups, of the dental and medical clinics of the institution (Alfenas, MG, Brazil), provided to the groups of patients and/or their parents-guardians, prior to the surgical rehabilitation procedures. The highest frequencies of oral colonization by *Candida* species were correlated with the group of infants and male sex. However, the frequencies of *Candida* species showed no significance for certain types of orofacial clefts or surgical history (Table 3), differently from prior observations (33). High rates of oral colonization by *Candida* species were observed in BCLP patients (*bilateral cleft lip and palate*: 77.7%) compared with UCLP and CP patients (*unilateral cleft lip and palate* and *cleft palate*: 57.1%). BCLP patients are more subject to hospitalizations and surgical procedures because of the deformities and the higher number of affected tissues, suggesting greater susceptibility to increased colonization by *Candida* species (33).

The prevalence of oral colonization (34–36) and vaginal colonization (36) by Candida species in elderly patients (34), patients with cancer (lung cancer, gastrointestinal tumor and hematopoietic systemic malignant tumor) (34–36) and HIV-soropositive patients (34–36) was found with no association with age or sex. However, patients with orofacial clefts compared with the control group (clinically healthy and

with no orofacial clefts) showed frequencies of *Candida* species and distribution of *C. albicans* variable according to age group (G1: ≤5 years of age; G2: ≥6 and ≤16 years of age; and G3: ≥17 years of age) (33). With increasing age, the frequency of non-albicans oral *Candida* species (*C. kefyr* and *C. glabrata*) increases regardless of the frequency of *C. albicans* in the oral cavity of both control patients (with no orofacial clefts and clinically healthy) and patients with orofacial clefts (33). These data partially corroborate our observations about the significantly higher frequencies of oral Candida species in groups of infants (0 to <2 years of age) (Table 3), and, also, a proportion of *C. albicans* and *Candida* spp. equivalent to 6:7 compared with the proportion of 1:3 for these species in groups of children (≥2 and <12 years of age) (Figure 1). These findings could be justified by the physiological and specific immunological systems (e.g., deciduous tooth eruption *versus* permanent dentition) in groups of patients of different age groups (i.e., infants and children).

Age-related physiological changes could partially justify this dynamics of colonization, since the natural barriers against the colonization of yeasts are established in body fluids and in mucosa and vary according to age. Additionally, the physiological succession of the species may be influenced by the eruption of deciduous and permanent teeth that lead to changes in the ecology of the oral cavity and habits of life. On the other hand, changes in the environment (hospital *versus* residence) in the diet (breastfeeding *versus* solid foods), and the use of soothers may represent other important factors (33,37,38). In addition, patients with orofacial clefts with at least three surgical histories showed higher rates of colonization by *Candida* species, suggesting that the events of hospitalizations and multiple surgeries generally expose patients to factors predisposing to increased colonization and infection by *Candida* species: broad-spectrum antibiotic therapy, treatment with corticosteroids, handling by health professionals carrying *Candida* spp., venous catheters, endotracheal anesthesia, nasogastric tubes and enteral nutrition (33). Although the correlation between the frequencies of *Candida* species and the surgical history is nonexistent in this research (Table 3), some of these factors above can partially contribute to our results and, also, suggest inefficiency in pre-surgical asepsis procedures: male infant (CLP15) showing oral colonization by *C. albicans* in period C and previous cheiloplasty; male child (CLP37) showing oral colonization by *C. albicans* in period C and previous cheiloplasties and palatoplasty; male infant (CLP43) showing oral colonization by *C. albicans* in periods A and C and previous cheiloplasty;

male infant (CLP45) showing oral colonization by *C. tropicalis* in periods A and C and without surgical history; male infant (CLP48) showing oral colonization by *C. albicans* in periods A and C and without surgical history (Figure 1 and Table 1).

The frequencies of *Candida* species in patients with orofacial clefts showed significantly reduced between the times of admission to surgical ward and prior to asepsis (period A) and immediately after surgical rehabilitation (period C), that is, partial and significant reduction in about 2 to 3 hours. These observations showed significant influence of asepsis with chlorhexidine, even under the effect of antibiotic therapy (first-generation cephalosporins: cefazolin or cephalotin) and surgical procedures (cheiloplasty and palatoplasty) (Figure 1 and Table 4). However, potential exogenous contamination, deficiencies in the containment barriers or low quality of pre-surgical asepsis could influence the relative reduction of oral colonization by *Candida* species. Therefore, further phylogenetic studies of those yeasts should be conducted to clarify these hypotheses and, in addition, efficient technical procedures for biosafety should be monitored and, if necessary, corrected.

Other interesting data showed an experimentally nonexistent incidence of *Candida* species in patients with orofacial clefts, considering the samplings immediately after asepsis with PVP-I or chlorhexidine (period B; 1–2 minutes post-asepsis) (Figure 1 and Table 5). The techniques employed to obtain the biological samples (swabs and PBS solution) associated with the antiseptic compounds residues generated (PVP-I or chlorhexidine), the time of transport and the laboratory processing could contribute to these findings. Routine hospital biosafety actions should be performed prior to surgical procedures. Thus, pre-operative asepsis of skin and mucous membranes can significantly reduce or eliminate their microbiota and further prevent occurrences of surgical site infections (SSI), according to the guidelines of the *Centers for Disease Control and Prevention* (CDC) (39) and *Association of periOperative Registered Nurses* (AORN) (40). Chlorhexidine and povidone-iodine are often used in surgical procedures, as pre-operative skin antiseptics, and recommended for application of surgical sites disinfections (41). Published systematic reviews and meta-analyses showed that skin asepsis with chlorhexidine had potential superiority over povidone-iodine in reducing rates of SSIs after clean or clean-contaminated surgery (42–45), chlorhexidine being preferably recommended (46).

For the groups of patients with orofacial clefts studied, there was a significant quantitative change (reduction of frequencies) and qualitative change (species-specific alterations) in the pattern of oral colonization by *Candida* species associated with the sampling times (periods A, B, C and D) (Figure 1 and Table 5). These results suggested the occurrence of a significant dynamic pattern of oral colonization by *Candida* species in patients with orofacial clefts over the pre- and post-operative periods. Such dynamics was characterized primarily by (*i*) elimination of *Candida* species after period A, (*ii*) elimination of *Candida* species after period A followed by oral neocolonization in period D, (*iii*) neocolonization by *Candida* species in period C or in period D, (*iv*) and neocolonization by *Candida* species in period C followed by maintenance and neocolonization in period D (Tables 5 and 6). These data report for the first time in Brazil and in the literature the pattern of oral colonization by *Candida* species before, during and after surgical rehabilitation procedures involving infants and children with different orofacial clefts.

An important aspect of the therapy against oral candidiasis is the elimination or treatment of underlying causes or identifiable risk factors (3). The treatment of oral candidiases depends on their early diagnosis, correction of facilitating factors or underlying diseases, clinical type (i.e., pseudomembranous, atrophic, hyperplastic and others) and proper use of antifungal agents, assessing the proportion between efficacy and toxicity in each individual case (47). The study of the antifungal susceptibility profile of *Candida* species (28) generates important epidemiological information and dental and medical implications for possible clinical and/or subclinical therapeutic conduct. In this research, all oral clinical isolates of *C. albicans* (n = 60), *C. krusei* (n = 60), *C. tropicalis* (n = 57) and *Candida* spp. (n = 5) showed profiles of susceptibility to amphotericin B (MIC < 1 μg/mL). Fluconazole resistance (MIC ≥ 64 μg/mL) occurred at low frequency (<10%) for oral clinical isolates of *C. albicans*, *C. krusei* and *C. tropicalis*, while dose-dependent susceptibility to fluconazole (MIC from 16–32 μg/mL) occurred at higher frequency (<50%) and exclusively for clinical isolates of *C. albicans*. Oral clinical isolates present variations in minimum inhibitory concentrations of nystatin (0.125 and 4 μg/mL; 0.61 and 19.43 IU), as expected in the literature (Table 7).

Amphotericin B is a polyene antifungal agent used in the treatment of localized fungal infections (ointment, cream or lotion) or systemic fungal infections (intravenous or oral suspension) (3). Cases of resistance to amphotericin B were generally rare in

treatments of invasive candidiasis. High frequencies of susceptibility to amphotericin B have been reported in the literature, using clinical isolates of *C. albicans* (92.8%) from various anatomical sites of hospitalized patients and surgical devices (48) and *Candida* species (96.3%: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. lusitaniae* and *Candida* spp.) from various anatomical sites of children with invasive candidiasis and surgical devices (32). Treatment with amphotericin B may be important especially in those cases of invasive candidiasis with resistance to fluconazole. Fluconazole is a triazole antifungal metabolized and excreted mainly by the kidneys. Its side effects are mild and include gastrointestinal discomfort and headache, with risk of hepatotoxicity (3) and potential multiple congenital fetal abnormalities from prolonged antifungal therapy in pregnant women (49). Variable rates of resistance to azole antifungals, particularly fluconazole, were reported for *Candida* species (i.e., *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *Candida* spp.), in different geographic regions, from hospitalized patients (50,51) and HIV-soropositive patients (52–54). Nystatin is another antifungal (polyene macrolide), derived from *Streptomyces* species, and targets fungal cell membranes, which contain ergosterol, an important sterol that controls the asymmetry and fluidity of cell membranes and is required so membrane-related enzymes function properly (55). The oral suspension may cause gastrointestinal discomfort, decreased glucose intolerance and increased risk for tooth decay (3). Effective actions of nystatin against *Candida* species have been reported, but its absorption does not occur in the gastrointestinal tract and intravenous administration is considerably toxic. Thus, the clinical therapy of nystatin is restricted to superficial infections (11). Nystatin doses recommended for topical use range from 200,000 to 600,000 IU (41.18 to 123.54 mg) for children and adults and 100,000 to 200,000 IU (20.59 to 41.18 mg) for newborns and infants, with therapeutic prescription of 1 to 4 weeks (56). Our results (0.125–4 μg/ml) potentially corroborate the success of clinically superficial antifungal therapy or prophylaxis in possible cases of nystatin prescription for patients with orofacial clefts with oral *Candida* species resistant to other antifungals.

Virulence tests of oral *Candida* species showed a phenotypic bias of the potential virulence mechanisms of those yeasts exerted on patients with orofacial clefts under pre- and post-surgical follow-up. Strongly positive activities of secreted aspartyl proteinases (SAP) occurred at high frequencies (≥90%) for the oral clinical isolates of *C. albicans*, *C. krusei*, *C. tropicalis* and *Candida* spp., while strongly positive activities of

phospholipases (PL) were infrequent (*C. krusei* and *Candida* spp.) or frequently low (36.7% for the isolates of *C. tropicalis* and 5.3% for the isolates of *C. tropicalis*) (Table 8). Thus, these results suggest that the potential partial activation of the virulence mechanisms of clinical isolates of *C. albicans* and *C.* non-*albicans* can be favored by the pre-existence of anatomical changes and tissue lesions, such as those found in patients with orofacial clefts, as well as the species-specific genetically intrinsic capacity. The capacity of *Candida* species to produce extracellular hydrolytic enzymes, such as *phospholipases* – PLs and *secreted aspartyl proteinases* – SAPs, has been considered as an important factor of virulence. PLs have an active role in lesions and tissue invasion, since their catalytic action results in disruption of epithelial cell membrane allowing the penetration of yeasts into host cell cytoplasm. While SAPs induce degradation of a variety of proteins, increasing the yeasts' capacity for colonization and penetration into host tissues (8). A trend similar to our findings was reported among clinical isolates of *C. albicans* (88.1% of SAP-positive isolates and 55.9% of PL-positive isolates) and *C.* non-*albicans* (69.8% of SAP-positive isolates and 37.7% of PL-positive isolates) isolated from samples from catheters, blood and oral cavity of HIV-soropositive patients (57). Other published investigations showed high rates of SAPs and PLs produced by clinical isolates of *C. albicans* and other species of the genus from diabetic individuals (8), patients with suspected invasive mycoses (58) and users of dental prostheses (19,59,60). Therefore, these last factors could be directly or indirectly associated with the activation mechanisms of both virulence factors: hydrolytic exoenzymes SAPs and PLs.

Conclusions

The occurrence of *Candida* species in Brazilian infants and children with orofacial clefts, in pre- and post-operative periods and until return to first consultation, were studied. A variable populational behavior was observed as to the first return of families and their children to the medical and dental unit, even under medical and dental guidelines, and which could be associated with socioeconomic, cultural and geographical factors, as well as to successful post-operative clinical outcomes. Some orofacial clefts presented preferably in male or female. Low incidence (39.1%) of oral colonization by *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis* and *Candida* spp.) was reported at patient admission to surgical ward, with no correlation to orofacial cleft types or

surgical history. The proper use of pre-operative antiseptics, especially chlorhexidine, associated with good hospital biosecurity habits could effectively eliminate colonizing fungal oral microbiota and contribute to the desired surgical clinical outcome. Over the pre-operative period, post-operative period and until return to first consultation (18 to 184 days, mean of 52.8 ±49.9 days), significant reduction in the frequencies of colonization by oral *Candida* species and species-specific alterations showed, for the first time in the literature, dynamic patterns of oral colonization: (*i*) complete elimination of *Candida species* at pre-operative period; (*ii*) elimination of *Candida* species at pre-operative period followed by neocolonization at return to first consultation; (*iii*) neocolonization by *Candida* species only at post-operative period or only at return to first consultation; and (*iv*) neocolonization by *Candida* species at post-operative period followed by species-specific maintenance and neocolonization at return to first consultation. In addition, these data confirmed the change of proportion of *C. albicans* and *C.* non-*albicans* over the development of patients with orofacial clefts. Despite this frequency having been considered low in the groups of infants and children with orofacial clefts studied, the *Candida* species were susceptible to amphotericin B and their susceptibility profiles in relation to nystatin were compatible with the clinical success of the prophylaxis and topical therapy. Some species (<10%) of *C. albicans*, *C. krusei* and *C. tropicalis* showed resistance to fluconazole; however, the dose-dependent susceptibility to fluconazole was reported in higher proportion (<50%) for clinical isolates of *C. albicans*. Certainly, such information could provide adequate therapeutic conduct concerning azole antifungals. Potentially and inherently virulent, some *Candida* species can modulate their patterns of hydrolytic exoenzyme activities, especially SAPs and PLs, according to the colonization and/or infection factors favored by the host's anatomical and immune nature.

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Transparency Declaration

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Figure 1. Experimental design and profile of oral colonization by *Candida* species in infants and children with orofacial clefts under surgical rehabilitation and clinical monitoring.

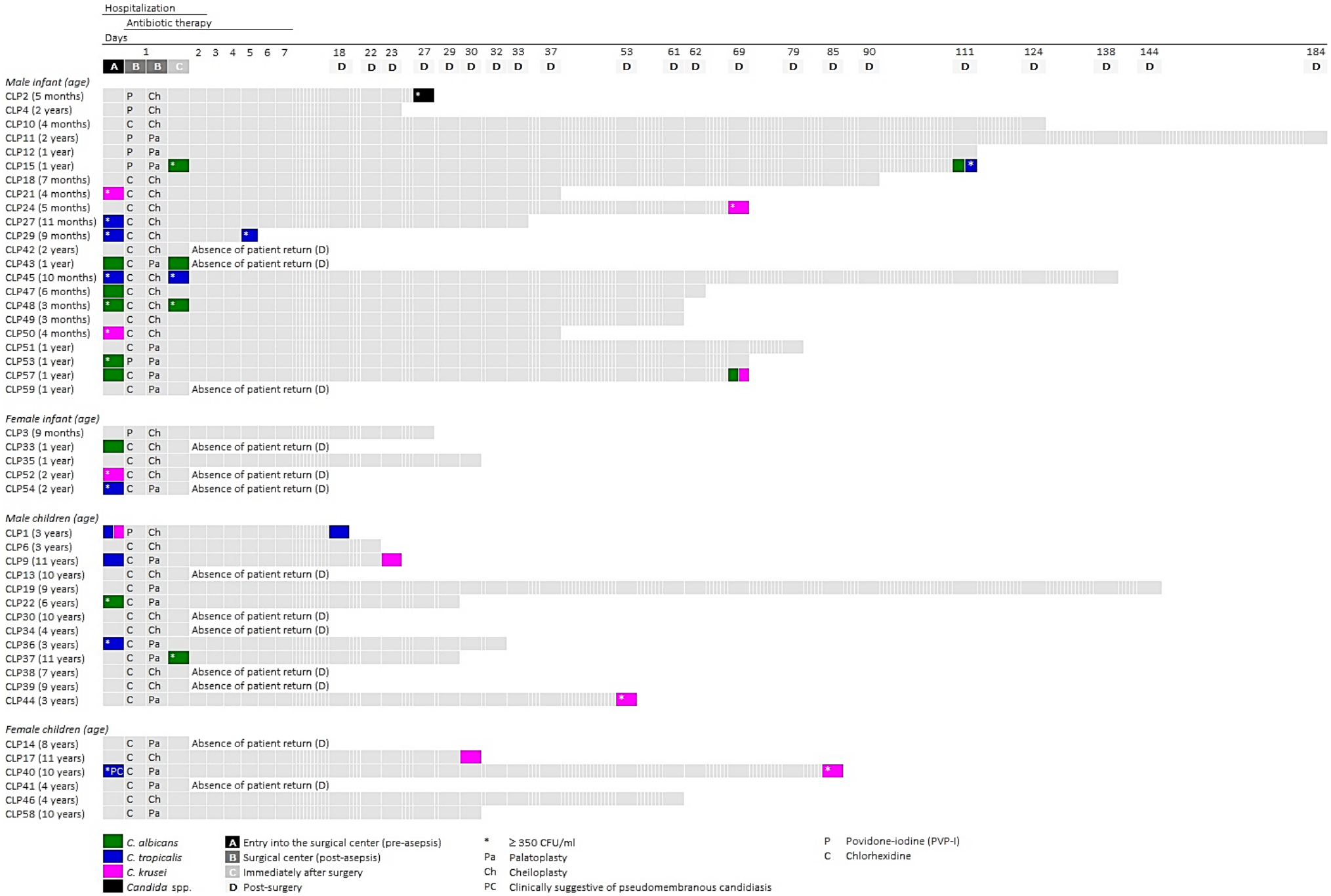
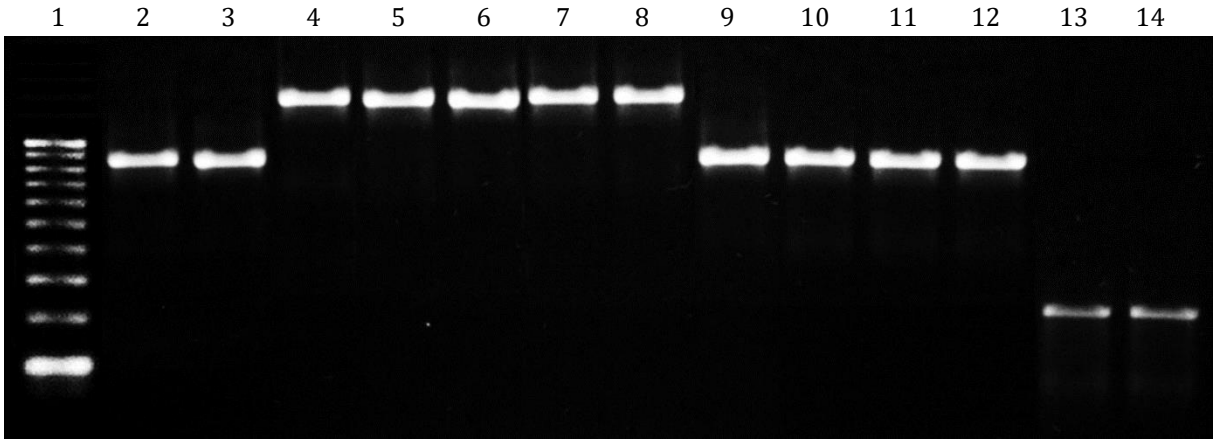


Figure 2. *Amplicons* of the PHR1 (1.644 bp) and DNA topolimerase II (860bp and 227bp) genes of the *C. albicans*, *C. krusei* and *C. tropicalis* clinical isolates, respectively, coming from oral cavity of the patients with orofacial clefts.



Agarose gel at 1.5% displaying stained amplicons with SYBR. Lane 1: DirectLoad™ PCR 100 bp Low Ladder, SIGMA Cat. # D3687; Lane 2: *type-strain* of *C. tropicalis* CBS 94; Lane 3: *clinical isolate of C. tropicalis* (CLP9); Lane 4: reference strain of *C. albicans* ATCC® 90028; Lane 5: *clinical isolate of C. albicans* (CLP22); Lane 6: *clinical isolate of C. albicans* (CLP33); Lane 7: *clinical isolate of C. albicans* (CLP43); Lane 8: *clinical isolate of C. albicans* (CLP47); Lane 9: *clinical isolate of C. tropicalis* (CLP27); Lane 10: *clinical isolate of C. tropicalis* (CLP29); Lane 11: *clinical isolate of C. tropicalis* (CLP40); Lane 12: *clinical isolate of C. tropicalis* (CLP54); Lane 13: *type-strain* of *C. krusei* CBS 573; Lane 14: *clinical isolate of C. krusei* (CLP21).

Table 1. Clinical classification of orofacial clefts and surgical clinical history of infants and children (SPINA, 1972).

Patient code (age)	Surgical history	Classification of the orofacial clefts
infant (11.2 ±6.6 months) — *n* = 27		
Male infant (10.2 ±6.2 months) — *n* = 22		
CLP2 (5 months)	-	Cleft pre-foramen left incomplete (CP-FLI)
CLP4 (<2 years)	Cheiloplasty (2013) and Palatoplasty (2014)	Cleft bilateral transforaminal (CBT)
CLP10 (4 months)	-	Cleft left transforamen (CLT)
CLP11 (<2 years)	Cheiloplasty (2014)	Cleft pre-foramen left incomplete (CP-FLI) / Cleft post-foramen incomplete (CP-FI)
CLP12 (1 year)	-	Cleft post-foramen incomplete (CP-FI)
CLP15 (1 year)	Cheiloplasty (2014)	Cleft transforamen right (CTR)
CLP18 (7 months)	-	Cleft transforamen right (CTR)
CLP21 (4 months)	-	Cleft transforamen right (CTR)
CLP24 (5 months)	-	Cleft left transforamen (CLT)
CLP27 (11 months)	-	Cleft bilateral transforaminal (CBT)
CLP29 (9 months)	-	Cleft transforamen right (CTR)
CLP42 (<2 years)	Cheiloplasty (2013) and Palatoplasty (2015)	Cleft pre-foramen right incomplete (CP-FRI) / Cleft post-foramen incomplete (CP-FI)
CLP43 (1 year)	Cheiloplasty (2015)	Cleft transforamen right (CTR)
CLP45 (10 months)	-	Cleft transforamen right (CTR) / Cleft pre-foramen left incomplete (CP-FLI)
CLP47 (6 months)	-	Cleft pre-foramen left incomplete (CP-FLI)
CLP48 (3 months)	-	Cleft left transforamen (CLT)
CLP49 (3 months)	-	Cleft pre-foramen left incomplete (CP-FLI)
CLP50 (4 months)	-	Cleft left transforamen (CLT) / Cleft pre-foramen right incomplete (CP-FRI)
CLP51 (1 year)	Cheiloplasty (2015)	Cleft left transforamen (CLT)
CLP53 (1 year)	-	Cleft post-foramen full (CP-FF)
CLP57 (1 year)	Cheiloplasty (2015)	Cleft transforamen right (CTR)
CLP59 (1 year)	Cheiloplasty (2015)	Cleft pre-foramen left incomplete (CP-FLI)
Female infant (15.8 ±6.7 months) — *n* = 5		
CLP3 (9 months)	-	Deformity groove-gingival (DG-G)
CLP33 (1 year)	-	Cleft pre-foramen left incomplete (CP-FLI) / Cleft post-foramen incomplete (CP-FI)
CLP35 (1 year)	Cheiloplasty (2015)	Cleft pre-foramen left incomplete (CP-FLI)
CLP52 (<2 year)	Cheiloplasty (2014)	Cleft pre-foramen left complete (CP-FLC)
CLP54 (<2 year)	Cheiloplasty (2014)	Cleft transforamen right (CTR)
Children (7.2 ±3.2 years old) — *n* = 19		
Male children (6.8 ±3.3 years old) — *n* = 13		
CLP1 (3 years)	Cheiloplasty (2013) and Palatoplasty (2014)	Cleft bilateral transforaminal (CBT)
CLP6 (3 years)	Cheiloplasty (2012 and 2013)	Cleft pre-foramen left complete (CP-FLC)
CLP9 (11 years)	Cheiloplasty (2005)	Cleft bilateral transforaminal (CBT)
CLP13 (10 years)	Cheiloplasty (2005 and 2009) and Palatoplasty (2006 and 2011)	Cleft transforamen right (CTR)
CLP19 (9 years)	Cheiloplasty (2006) and Palatoplasty (2011 and 2013)	Cleft bilateral transforaminal (CBT)
CLP22 (6 years)	-	Cleft post-foramen incomplete (CP-FI)
CLP30 (10 years)	-	Cleft pre-foramen right incomplete (CP-FRI)
CLP34 (4 years)	Cheiloplasty (2012) and Palatoplasty (2013)	Cleft pre-foramen right incomplete (CP-FRI) / Cleft post-foramen incomplete (CP-FI)
CLP36 (3 years)	Cheiloplasty (2013) and Palatoplasty (2015)	Cleft left transforamen (CLT)
CLP37 (11 years)	Cheiloplasty (2005 and 2011) and Palatoplasty (2005)	Cleft transforamen right (CTR)
CLP38 (7 years)	Cheiloplasty (2009 and 2015) and Palatoplasty (2010 and 2014)	Cleft left transforamen (CLT)
CLP39 (9 years)	Cheiloplasty (2007) and Palatoplasty (2009 and 2013)	Cleft left transforamen (CLT) / Cleft pre-foramen right incomplete (CP-FRI)
CLP44 (3 years)	Cheiloplasty (2014) and Palatoplasty (2015)	Cleft transforamen right (CTR)
Female children (7.8 ±3.1 years old) — *n* = 6		
CLP14 (8 years)	Palatoplasty (2008, 2009 and 2011)	Cleft post-foramen incomplete (CP-FI)
CLP17 (11 years)	-	Cleft pre-foramen left complete (CP-FLC)
CLP40 (10 years)	-	Cleft left transforamen (CLT)
CLP41 (4 years)	Palatoplasty (2013 and 2015)	Cleft post-foramen incomplete (CP-FI)
CLP46 (4 years)	Cheiloplasty (2012)	Cleft pre-foramen full right (CP-FFR)
CLP58 (10 years)	-	Cleft submucosa post-foramen incomplete (CSP-FI)

Table 2. Clinical classification of the orofacial clefts between groups (infants and children) or gender (male and female) of patients.

Classification of the orofacial clefts	Infants		Children		∑		*p*-valor
	N	%	*n*	%	*n*	%	
Cleft bilateral transforaminal	2	6.3	3	14.3	5	9.4	0.11
Cleft left transforamen	5	15.6	4	19.0	9	17.0	0.73
Cleft post-foramen full	1	3.1	-	-	1	1.9	-
Cleft post-foramen incomplete	4	12.5	4	19.0	8	15.1	0.28
Cleft pre-foramen full right	-	-	1	4.8	1	1.9	-
Cleft pre-foramen left complete	1	3.1	2	9.5	3	5.7	0.09
Cleft pre-foramen left incomplete	8	25.0	-	-	8	15.1	-
Cleft pre-foramen right incomplete	2	6.3	3	14.3	5	9.4	0.12
Cleft submucosa post-foramen incomplete	-	-	1	4.8	1	1.9	-
Cleft transforamen right	8	25.0	3	14.3	11	20.8	0.1
Deformity groove-gingival	1	3.1	-	-	1	1.9	-
∑	32	100	21	100	53	100	
Classification of the orofacial clefts	Female		Male		∑		
	N	%	*n*	%	*n*	%	
Cleft bilateral transforaminal	-	-	5	12.2	5	9.4	-
Cleft left transforamen	1	8.3	8	19.5	9	17.0	0.03*
Cleft post-foramen full	-	-	1	2.4	1	1.9	-
Cleft post-foramen incomplete	3	25.0	5	12.2	8	15.1	0.04*
Cleft pre-foramen full right	1	8.3	-	-	1	1.9	-
Cleft pre-foramen left complete	2	16.7	1	2.4	3	5.7	0.001**
Cleft pre-foramen left incomplete	2	16.7	6	14.6	8	15.1	0.8
Cleft submucosa post-foramen incomplete	1	8.3	-	-	1	1.9	-
Cleft pre-foramen right incomplete	-	-	5	12.2	5	9.4	-
Cleft transforamen right	1	8.3	10	24.4	11	20.8	0.008*
Deformity groove-gingival	1	8.3	-	-	1	1.9	-
∑	12	100	41	100	53	100	

* Statistically significant differences for value $p < 0.05$. ** Statistically significant differences for value $p < 0.001$

Table 3. Incidence of oral *Candida* species coming from patients with orofacial clefts during admission to the surgical center (prior to asepsis: period A).

Correlation groups	Patients with orofacial clefts		Subjects carrying oral *Candida* species		*Candida* species	*p*-valor
	n	%	*n*	%		
Cleft bilateral transforaminal	5	9.4	3	5.7	*C. tropicalis* (*n* = 3; 5.8%)	-
Cleft left transforamen	9	17.0	4	7.5	*C. albicans* (*n* = 1; 1.9%), *C. krusei* (*n* = 1; 1.9%) and *C. tropicalis* (*n* = 2; 3.8%)	-
Cleft post-foramen full	1	1.9	1	1.9	*C. albicans* (*n* = 1; 1.9%)	-
Cleft post-foramen incomplete	8	15.1	2	3.8	*C. albicans* (*n* = 2; 3.8%)	-
Cleft pre-foramen full right	1	1.9	-	-	-	-
Cleft pre-foramen left complete	3	5.7	1	1.9	*C. krusei* (*n* = 1; 1.9%)	-
Cleft pre-foramen left incomplete	8	15.1	3	5.7	*C. albicans* (*n* = 2; 3.8%), and *C. tropicalis* (*n* = 1; 1.9%)	-
Cleft pre-foramen right incomplete	5	9.4	1	1.9	*C. krusei* (*n* = 1; 1.9%)	-
Cleft submucosa post-foramen incomplete	1	1.9	-	-	-	-
Cleft transforamen right	11	20.8	6	11.3	*C. albicans* (*n* = 2; 3.8%), *C. krusei* (*n* = 1; 1.9%), and *C. tropicalis* (*n* = 3; 5.8%)	-
Deformity groove-gingival	1	1.9	-	-	-	-
∑	53	100	21	39.6		
Obs.: There are 7 subjects with two classification of the cleft lip and palate.						
Infants**	27	58.7	13	28.3**	*C. albicans* (*n* = 6; 13.0%), *C. krusei* (*n* = 3; 6.5%), and *C. tropicalis* (*n* = 4; 8.7%)	0.0005**
Children	19	41.3	5	10.9	*C. albicans* (*n* = 1; 2.2%), *C. krusei* and *C. tropicalis* (*n* = 1; 2.2%), and *C. tropicalis* (*n* = 3; 6.5%)	0.0001**
∑	46	100	18	39.1		
Female	11	23.9	4	8.7	*C. albicans* (*n* = 1; 2.2%), *C. krusei* (*n* = 1; 2.2%), and *C. tropicalis* (*n* = 2; 4.3%)	0.01*
Male*	35	76.1	14	30.4*	*C. albicans* (*n* = 6; 13.0%), *C. krusei* (*n* = 2; 4.3%), *C. tropicalis* (*n* = 5; 10.9%), and *C. krusei* and *C. tropicalis* (*n* = 1; 2.2%)	0.0001**
∑	46	100	18	39.1		
With surgical history	25	54.3	7	15.2	*C. albicans* (*n* = 2; 4.3%), *C. krusei* (*n* = 1; 2.2%), *C. tropicalis* (*n* = 3; 6.5%), and *C. krusei* and *C. tropicalis* (*n* = 1; 2.2%)	0.0001**
Without surgical history	21	45.7	11	23.9	*C. albicans* (*n* = 5; 10.8%), *C. krusei* (*n* = 2; 4.3%), and *C. tropicalis* (*n* = 4; 8.7%)	0.01*
∑	46	100	18	39.1		
Cheiloplasty	12	33.3	5	13.9	*C. albicans* (*n* = 2; 5.6%), *C. krusei* (*n* = 1; 2.7%), and *C. tropicalis* (*n* = 2; 5.6%)	0.008*
Palatoplasty	2	5.6	-	-	-	-
Cheiloplasty and Palatoplasty	22	61.1	2	5.6	*C. krusei* and *C. tropicalis* (*n* = 1; 2.7%), and *C. tropicalis* (*n* = 1; 2.7%)	0.0001**
∑	36	100	7	19.4		

* Statistically significant differences for value $p < 0.05$. ** Statistically significant differences for value $p < 0.001$

Table 4. Incidence of oral *Candida* species coming from patients with orofacial clefts comparatively between the admission period to the surgical center (prior to asepsis: period A) and immediately after surgical rehabilitation (period C).

Patients with orofacial clefts	Before asepsis (period A)			Immediately after surgery (period C)			*p*-valor
	n	%	Species	*n*	%	Species	
Chlorhexidine (*n* = 38 subjects)	16	42.1	*C. albicans* (*n* = 6; 15.8%), *C. krusei* (*n* = 3; 7.9%) and *C. tropicalis* (*n* = 7; 18.4%)**	4 (being 1 from prior absence)	10.5	*C. albicans* (*n* = 3; 7.9%) and *C. tropicalis* (*n* = 1; 2.6%)**	0.001**
PVP-I (*n* = 8 subjects)	2	25.0	*C. albicans* (*n* = 1; 12.5%), and *C. krusei* and *C. tropicalis* (*n* = 1; 12.5%)**	1 (being 1 from prior absence)	12.5	*C. albicans* (*n* = 1; 12.5%)**	0.07
Cheiloplasty (*n* = 27 subjects)	10	37.0	*C. albicans* (*n* = 3; 11.1%), *C. krusei* (*n* = 3; 11.1%), *C. tropicalis* (*n* = 3; 11.1%), and *C. krusei* and *C. tropicalis* (*n* = 1; 3.7%)	2	7.4	*C. albicans* (*n* = 1; 3.7%) and *C. tropicalis* (*n* = 1; 3.7%)*	0.0001**
Palatoplasty (*n* = 19 subjects)	8	42.1	*C. albicans* (*n* = 4; 21.1%) and *C. tropicalis* (*n* = 4; 21.1%)	3 (being 2 from prior absence)	15.8	*C. albicans* (*n* = 3; 15.8%)*	0.006*
Antibiotic therapy (*n* = 46 subjects)	18	39.1	*C. albicans* (*n* = 7; 15.2%), *C. krusei* (*n* = 3; 6.5%), *C. tropicalis* (*n* = 7; 15.2%), and *C. krusei* and *C. tropicalis* (*n* = 1; 2.2%)	5	10.9	*C. albicans* (*n* = 4; 8.7%) and *C. tropicalis* (*n* = 1; 2.2%)	0.0001**

* Statistically significant differences for value $p < 0.05$. ** Statistically significant differences for value $p < 0.001$

Table 5. Incidence of oral *Candida* species coming from patients with orofacial clefts comparatively between the admission period to the surgical center (prior to asepsis: period A), immediately after asepsis (period B), immediately after surgical rehabilitation (period C), and return of the patient after surgery (period D: ≥5 and ≤184 days; mean of 52.8 ±49.9 days).

Sampling period	Patients with orofacial clefts		Patients carrying oral *Candida* species		*Candida* species
	n	%	*n*	%	
Entry into the surgical center (pre-asepsis) – Period A	46	100	18	39.1	*C. albicans* (*n* = 7; 15.2%), *C. krusei* (*n* = 3; 6.5%), *C. tropicalis* (*n* = 7; 15.2%), *C. krusei* and *C. tropicalis* (*n* = 1; 2.2%)
Surgical center (post-asepsis) – Period B	46	100	-	-	-
Immediately after surgery – Period C	46	100	5	10.9	*C. albicans* (*n* = 4; 8.7%) and *C. tropicalis* (*n* = 1; 2.2%)
Post-surgery – Period D	33	71.7	10	21.7	*C. albicans* and *C. tropicalis* (*n* = 1; 2.2%), *C. albicans* and *C. krusei* (*n* = 1; 2.2%), *C. krusei* (*n* = 5; 10.9%), *C. tropicalis* (*n* = 2; 4.3%), and *Candida* spp. (*n* = 1; 2.2%)
value *p*		-		0.0001	

* Statistically significant differences for value $p < 0.001$.

Table 6. Dynamics and frequencies of oral colonization by *Candida* species coming from patients with orofacial clefts throughout the pre- and post-surgical periods.

Dynamics of oral colonization by *Candida* species	*n*	%
Absence of *Candida* species in the experimental period (ABCD and ABC)	22	47.8
Elimination in period A (*C. albicans*: *n* = 4, 8.7%; *C. krusei*: *n* = 3, 6.5%; *C. tropicalis*: *n* = 3, 6.5%)	10	21.7
Elimination in period A and neocolonization in period D (C. tropicalis → *C. tropicalis*: *n* = 2, 4.3%; *C. tropicalis* → *C. krusei*: *n* = 2, 4.3%; *C. albicans* → *C. albicans* and *C. krusei*: *n* = 1, 2.2%)	5	10.9
Maintenance in period A and C (*C. albicans*: *n* = 2, 4.3%; *C. tropicalis*: *n* = 1, 2.2%)	3	6.5
Neocolonization in period C (*C. albicans*: *n* = 1, 2.2%) or period D (*C. krusei*: *n* = 3, 6.5%; *Candida* spp.: *n* = 1, 2.2%)	5	10.9
Neocolonization in period C (*C. albicans: n* = 1, 2.2%) and maintenance and neocolonization in period D (*C. albicans* and *C. tropicalis*)	1	2.2
∑	46	100
value *p*	-	0.0001

* Statistically significant differences for value $p < 0.001$.

Table 7. Antifungal sensitivity profile (amphotericin B, fluconazole and nystatin) of *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis*, and *Candida* spp.) isolated from the oral cavity of patients with orofacial clefts.

Antifungals	MIC	*C. albicans*		*C. krusei*		*C. tropicalis*		*Candida* spp.	
		N	%	*n*	%	*n*	%	*n*	%
Amphotericin B	< 1 μg/ml (S)	60	100.0	60	100.0	57	100.0	5	100.0
	≥ 1 μg/ml (R)	-	-	-	-	-	-	-	-
	∑	60	100.0	60	100.0	57	100.0	5	100.0
Fluconazole	< 8 μg/ml (S)	29	48.3	55	91.7	54	94.7	5	100.0
	16-32 μg/ml (SDD)	29	48.3	-	-	-	-	-	-
	≥ 64 μg/ml (R)	2	3.3	5	8.3	3	5.3	-	-
	∑	60	100.0	60	100.0	57	100.0	5	100.0
Nystatin	4 μg/ml	-	-	6	10.0	-	-	-	-
	2 μg/ml	2	3.3	2	3.3	-	-	-	-
	1 μg/ml	32	53.3	29	48.3	24	42.1	2	40.0
	0.5 μg/ml	26	43.3	22	36.7	33	57.9	3	60.0
	0.25 μg/ml	-	-	-	-	-	-	-	-
	0.125 μg/ml	-	-	1	1.7	-	-	-	-
	∑	60	100.0	60	100.0	57	100.0	5	100.0

Table 8. Enzyme activity indexes (*Pz*) of secreted aspartyl proteinase (SAP) and phospholipases (PL) from the clinical isolates of *Candida* species isolated from the oral cavity of patients with orofacial clefts.

Hydrolytic enzyme	Enzymatic activity	*C. albicans*		*C. krusei*		*C. tropicalis*		*Candida* spp.	
		N	%	*n*	%	*n*	%	*n*	%
Secreted aspartyl proteinases (SAP)	*Pz* = 0	3	5.0	6	10.0	1	1.8	-	-
	Pz = 1	-	-	-	-	-	-	-	-
	Pz = 2	57	95.0	54	90.0	56	98.2	5	100.0
	∑	60	100.0	60	100.0	57	100.0	5	100.0
Phospholipase (PL)	*Pz* = 0	38	63.3	60	100.0	54	94.7	5	100.0
	Pz = 1	-	-	-	-	-	-	-	-
	Pz = 2	22	36.7	-	-	3	5.3	-	-
	∑	60	100.0	60	100.0	57	100.0	5	100.0

3 CONCLUSÃO

O conjunto de dados obtidos e sistematicamente analisados, a partir do desenvolvimento da pesquisa científica intitulada “Biótipos de espécies de *Candida* na cavidade bucal de crianças portadoras de fissuras labial e palatina”, permite inferir as seguintes conclusões:

1) Durante a amostragem biológica bucal, envolvendo os pacientes portadores de fissuras orofaciais, observa-se que a maioria daqueles pacientes retornou à primeira consulta médica para a avaliação clínica e laboratorial, considerando uma ampla variação no período de retorno. Portanto, especula-se que fatores socioeconômicos, culturais e geográficos podem influenciar na conduta dos pacientes estudados.

2) Entre as fissuras orofaciais identificadas no estudo, nenhuma classificação mostra-se exclusivamente estratificada em um determinado grupo de pacientes (bebês ou crianças). Por outro lado, algumas classificações de fissuras orofaciais podem apresentar-se preferencialmente nos sexos masculino ou feminino.

3) No período de admissão no centro cirúrgico e previamente à assepsia (período A), uma parcela dos grupos de pacientes com fissuras orofaciais (39,1%) apresenta colonização bucal mista e/ou homogênea por espécies de *Candida*, especialmente por *C. albicans*, *C. krusei* e *C. tropicalis*, independentemente dos tipos de fissuras orofaciais ou histórico cirúrgico. Pacientes do sexo masculino e bebês revelam maior frequência à colonização por *Candida*. Entretanto, observa-se casos raros de suspeita clínica para candidose pseudomembranosa que devem ser diagnosticados e tratados.

4) As frequências das espécies de *Candida* nos pacientes portadores de fissuras orofaciais mostram-se significativamente reduzida entre os momentos de admissão no centro cirúrgico (previamente à assepsia) e imediatamente após a reabilitação cirúrgica. Estas observações mostram forte influência da assepsia com clorexidina, mesmo sob o efeito da antibiótico terapia e os procedimentos

cirúrgicos. Potencial contaminação exógena e a baixa qualidade da assepsia pré-cirúrgica podem influenciar na redução relativa de colonização bucal por espécies de *Candida*.

5) Menores frequências das espécies de *Candida* (quantitativas e qualitativas) nos pacientes portadores de fissuras orofaciais estão associadas significativamente com os momentos antecedendo o procedimento cirúrgico e imediatamente após a assepsia com PVP-I ou clorexidina (período B) e imediatamente após a reabilitação cirúrgica (período C), seguido do primeiro retorno do paciente ao centro médico (período D), em comparação com o momento da admissão do paciente no centro cirúrgico e previamente à assepsia (período A).

6) Um padrão dinâmico significativo de colonização bucal por espécies de *Candida* ocorre em pacientes portadores de fissuras orofaciais ao longo dos períodos pré- e pós-operatório. Tal dinâmica é caracterizada principalmente pela (*i*) eliminação das espécies de *Candida* posterior ao período A, (*ii*) eliminação das espécies de *Candida* posterior ao período A seguido da neocolonização bucal no período D, (*iii*) neocolonização por espécies de *Candida* no período C ou no período D, (*iv*) e neocolonização por espécies de *Candida* no período C seguido de manutenção e neocolonização no período D.

7) O estudo do perfil de sensibilidade antifúngica das espécies de *Candida* gera importantes implicações médicas e odontológicas para uma eventual conduta terapêutica clínica e/ou subclínica. Todos os isolados clínicos bucais de *C. albicans*, *C. krusei*, *C. tropicalis* e *Candida* spp. mostram-se sensíveis à anfotericina B. A resistência ao fluconazol ocorre em frequência baixa (<10%) para os isolados clínicos bucais de *C. albicans*, *C. krusei* e *C. tropicalis*, enquanto que a sensibilidade dose dependente ao fluconazol ocorre em frequência maior (<50%) e exclusivamente para os isolados clínicos de *C. albicans*. Isolados clínicos bucais apresentam variações nas concentrações inibitórias mínimas da nistatina (0,125 e 4 μg/mL), conforme esperado na literatura.

8) Os ensaios de potencial de virulência das espécies bucais de *Candida* revelam uma parcialidade fenotípica dos mecanismos de virulência em pacientes com fissuras orofaciais sob acompanhamento pré- e pós-cirúrgico. Atividades fortemente positivas de aspartil proteinases secretadas (APS) ocorrem em altas frequências (≥90%) para os isolados clínicos bucais de *C. albicans*, *C. krusei*, *C. tropicalis* e *Candida* spp., enquanto que as atividades fortemente positivas de fosfolipases (FL) são infrequentes (*C. krusei* e *Candida* spp.) ou frequentemente baixas (*C. albicans* e *C. tropicalis*).

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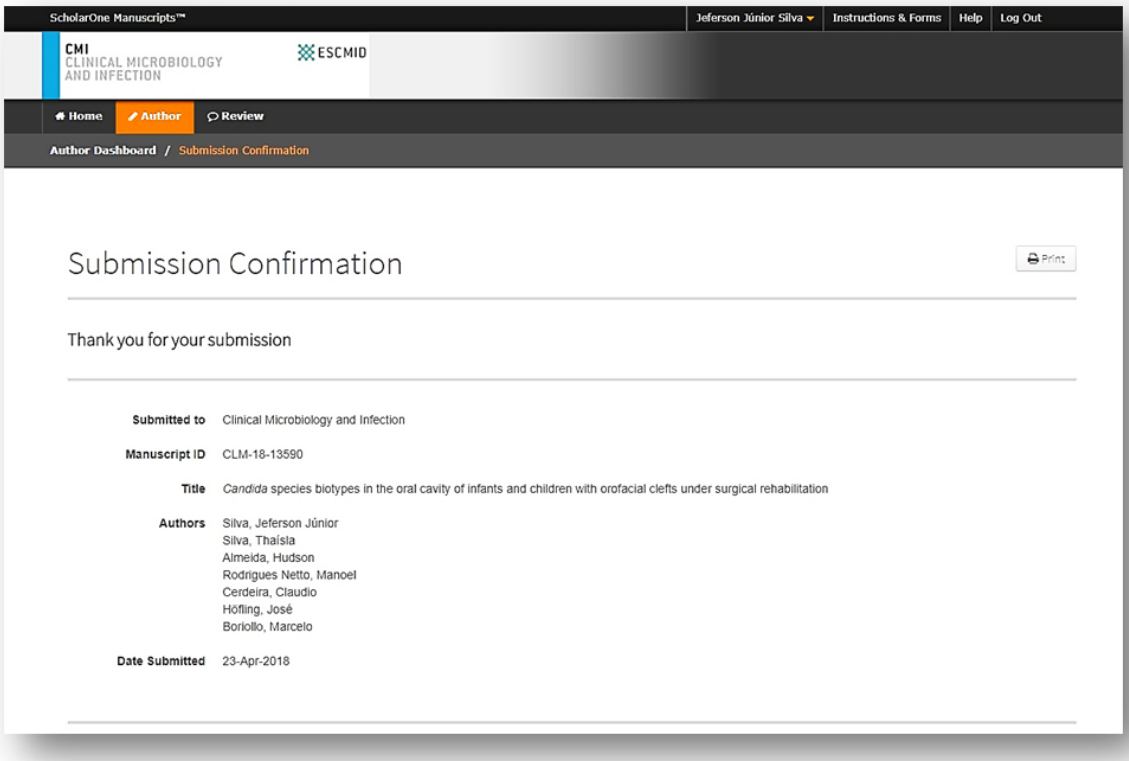
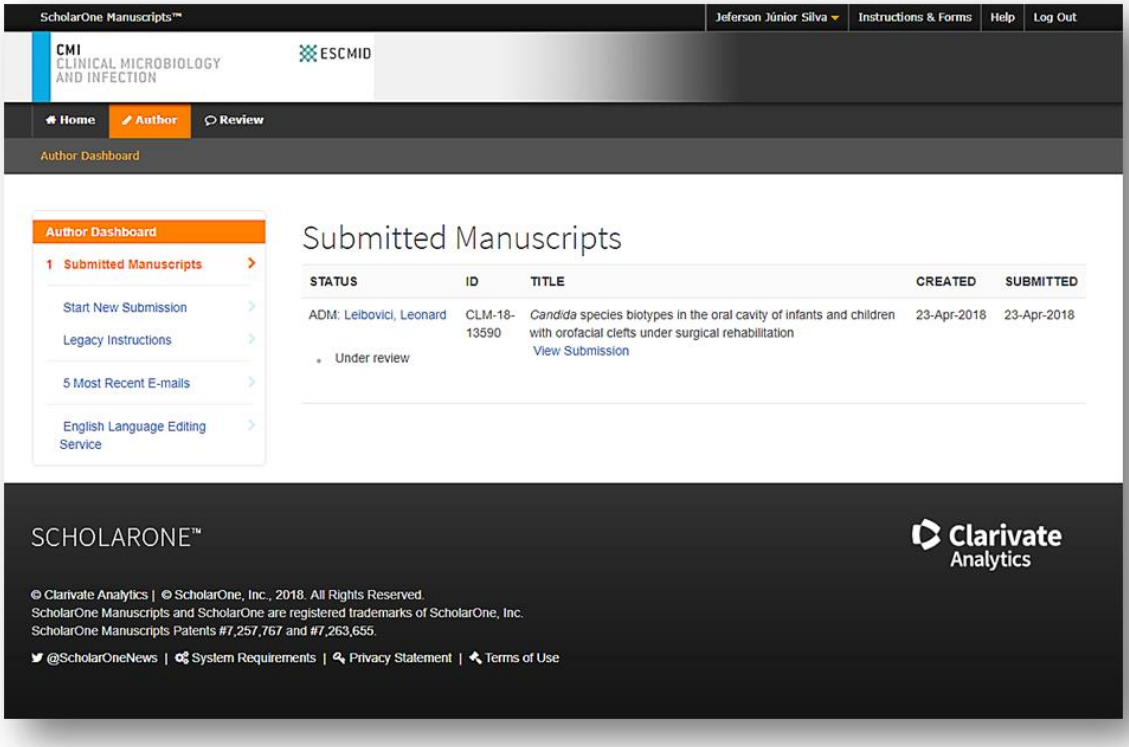
ANEXOS

Anexo 1 – Comprovante de submissão do trabalho.

https://www.journals.elsevier.com/clinical-microbiology-and-infection/





Anexo 2 – Certificação do Comitê de Ética

25/01/2018 Comitê de Ética em Pesquisa - Certificado

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Polimorfismo de DNA microssatélite, virulência in vitro e sensibilidade antifúngica e fitoterápica (Anacardium occidentale, Paullinia cupana e Euterpe oleracea) de Candida albicans proveniente de crianças portadoras de fissuras labial e palatina"**, protocolo nº 093/2014, dos pesquisadores Marcelo Fabiano Gomes Boriollo, Jeferson Junior da Silva e Manoel Francisco Rodrigues Netto, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/12/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Polymorphism of microsatellite DNA, in vitro virulence and antifungal and phytotherapic sensitivity (Anacardium occidentale, Paullinia cupana and Euterpe oleracea) of Candida albicans from children with cleft lip and palate"**, register number 093/2014, of Marcelo Fabiano Gomes Boriollo, Jeferson Junior da Silva and Manoel Francisco Rodrigues Netto, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Dec 12, 2014.

Prof. Dr. Jacks Jorge Junior
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Felippe Bevilacqua Prado
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

http://w2.fop.unicamp.br/cep/sistema/certificado.php?Protocolo=093/2014&Id=2243&Passo=2&DataPar=2014-12-12 1/1